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# CANCER RESEARCH

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CANCER RESEARCH

VOLUME 10 JANUARY 1950 NUMBER 1

# Studies on the Relationship between Sarcoma and Leukosis in Chickens

## II. Histogenesis of Tumors Induced by Intramuscular Inoculation of Cell-containing Leukotic Material

M. PIKOVSKI AND L. DOLJANSKI*

*(From the Department of Experimental Pathology Cancer Laboratories, The Hebrew University, Jerusalem, Israel)*

In a previous paper we gave an account of the results of intramuscular inoculations of cell-free and cell-containing leukotic material of Strain T Engelbreth-Holm (hemocytoblastosis) into fowls (1).

We showed that tumor was never produced at the site of intramuscular inoculation of cell-free leukotic material (plasma, irradiated liver). On the other hand inoculations of cell-containing material (blood, non-irradiated liver) regularly resulted in a tumor at the site of inoculation when suitable material and methods were employed. The leukotic agent was active in both cases since almost all chickens inoculated with cell-containing as well as with cell-free material died of leukemia. It was evident that the appearance of the tumor was not due to the action of the agent on the connective tissue cells but depended on the presence of hemocytoblasts in the inoculated material.

A study of cultures of leukotic blood cells and of bone marrow of the same strain *in vitro* (2) and of the tumors following intramuscular inoculation of hemocytoblasts showed that in both cases hemocytoblasts became transformed into fibroblasts. This paper deals with the histology and histogenesis of the tumors.

* Deceased, April 13, 1948.

Received for publication, July 29, 1949.

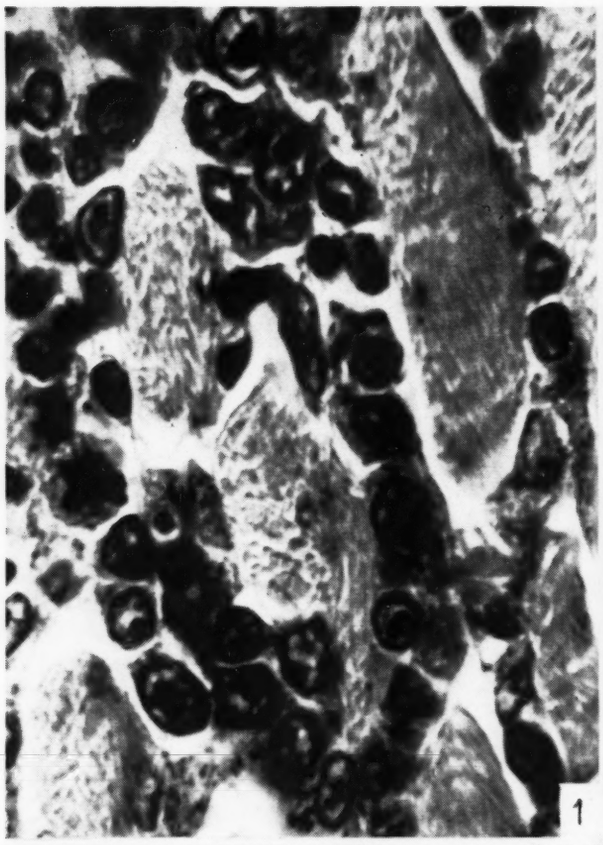
### EXPERIMENTS

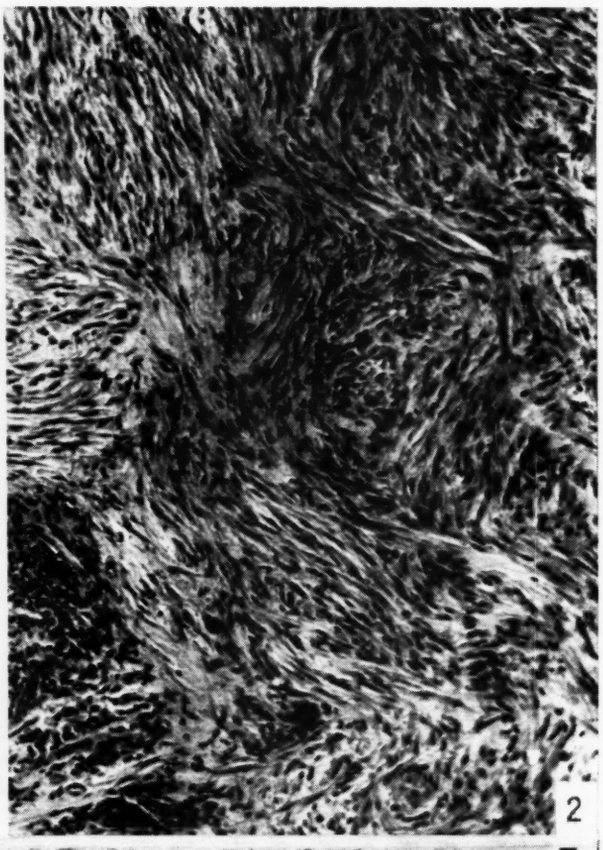
In the first series of experiments whole blood or a diluted suspension of hemocytoblasts was injected intramuscularly. In the second series of experiments leukotic liver in which the capillaries were packed with hemocytoblasts was inoculated intramuscularly. The inoculation resulted in tumor in 22 out of 26 inoculations. Of the 4 chickens which did not develop tumor, 3 did not develop leukemia. The tumors produced by inoculation of blood or liver were macroscopically and microscopically of the same type.

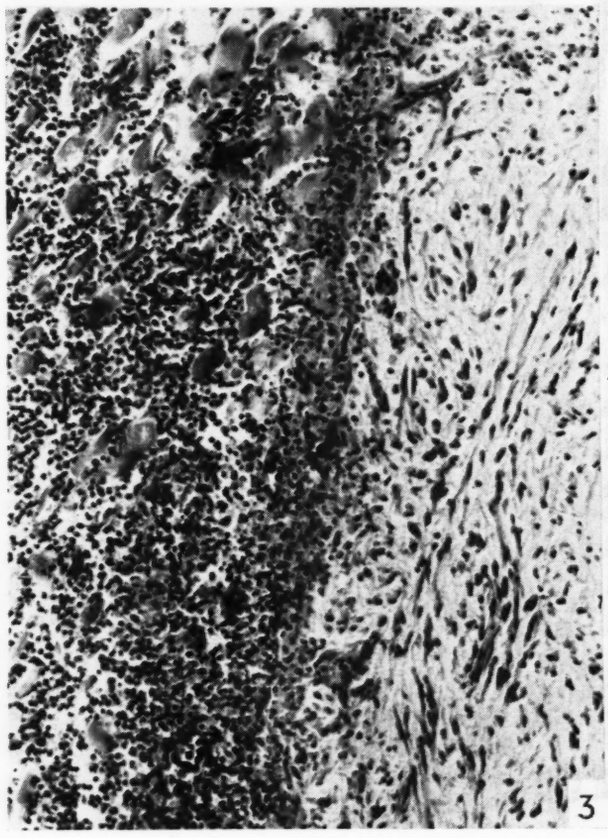
### DESCRIPTION OF TUMORS

*Gross Appearance.*—The tumors were either nodular, varying in size from lentil to small hazelnut, or flat. In the latter case they were felt as a swelling of the whole pectoral muscle. Hemorrhages were seen around some tumors. Sometimes no tumor could be palpated but on post mortem examination either hemorrhages or long white streaks were detected in the muscles. The tumors did not produce metastases and in no case did we succeed in passaging them more than 3 times.

*Microscopic Findings.*—Our findings correspond in some respects with those of Engelbreth-Holm and collaborators on tumors produced by intramuscular inoculations of the same strain (3). They also resemble those of Jarmai who described a tumor







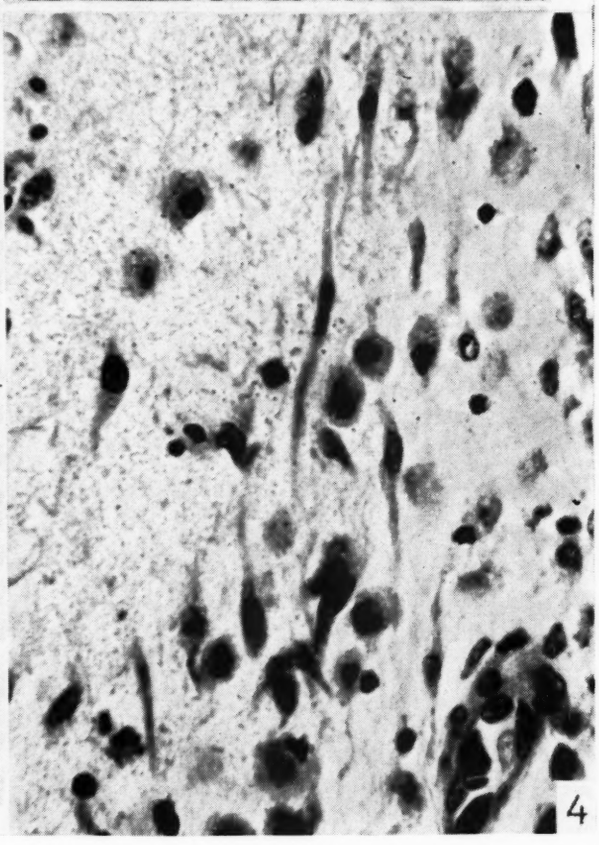


FIG. 1.—Invasion of muscle tissue by hemocytoblasts in the external zone of the tumor. Mag. ×1400.

FIG. 2.—Central zone of a tumor, resembling a fibrosarcoma. Mag. ×180.

FIG. 3.—The boundary between the outer round cell zone and the inner spindle cell zone of a tumor showing more and less elongated cells of different shapes. Mag. ×180.

FIG. 4.—The same area with higher magnification. Mag. ×670.

which appeared at the site of inoculation of liver from a fowl infected with his leukotic strain of hemocytoblastosis (4).

In our experiments all the tumors showed the same general pattern with constant characteristic features. Individual tumors varied in so far as one or the other feature predominated. The usual picture was as follows: In the muscle tissue adjacent to the tumor masses of hemocytoblasts were scattered. In some areas many hemocytoblasts were undergoing disintegration, showing different degrees of pyknosis and karyolysis. Adjacent to foci of disintegrating cells there were areas of actively multiplying hemocytoblasts which invaded the muscle tissue in long streaks, infiltrating between the muscle fibres. These were large round or polygonal cells with markedly basophilic protoplasm and a big centrally located nucleus (Fig. 1). Mitoses were often encountered. The central part of the tumor usually consisted of more or less densely packed bundles of spindle cells and it often resembled a fibrosarcoma (Fig. 2). On the boundary between the outer round cell zone and the inner fibroblast zone (Fig. 3) elongated hemocytoblasts (long cells with a typical hemocytoblast nucleus) were frequently present (Fig. 4). They looked very much like those seen by us *in vitro* when cultures of hemocytoblasts were in process of transforming themselves into cultures of fibroblasts (Figs. 5, 6).

Many cells were of a spindle cell type: they were elongated pale cells with ampholitic cytoplasm, an oval nucleus poor in chromatin, usually two nucleoli, and a rather well pronounced nuclear wall which was often uneven or indented.

In the loosely arranged tissue of such an area among the round and elongated stem cells and newly formed fibroblasts were scattered many big pale ameboid cells, rounded or elongated, with an eccentric nucleus. They were often filled with phagocytosed debris. These cells of a macrophage type were numerous in cultures of leukotic cells and were described by us as polyblasts (Fig. 6). *In vitro*, the transformation of stem cells to polyblasts and of polyblasts to fibroblasts could be easily followed. As *in vitro* the fibroblasts deriving from the inoculated hemocytoblasts proliferated actively. It could be clearly observed that the spindle cells made their way between the muscle fibres, pressing them apart and separating them. The fibroblasts build a new tissue which sometimes invades the muscle, always from the inner part of the tumor nodule. Mitoses are often seen in fibroblasts.

In the immediate vicinity of hemorrhages, which were often seen around tumors, the tissue consisted of loosely arranged hemocytoblasts, elongated hemocytoblasts, and long cells of endothelial type. Between those loosely arranged cells many capillaries, probably newly formed, were often observed. This tissue resembles an angiosarcoma (Fig. 7). It is interesting to note that in the instances when at the site of inoculation no tumor could be palpated and only hemorrhages or white streaks in the muscle were observed on post mortem, the microscopic picture was always one of a muscle tissue invaded by fibroblasts and associated with angiosarcoma type tissue.

Nodules of different dimensions, consisting of densely packed lymphoid cells, were frequently found in the tumor tissue. Most of those cells were of the lymphocyte type but many hemocytoblasts could also be recognized. Such nodules were also frequently found in the muscle in the vicinity of the tumor (Fig. 9). They were always surrounded by multinuclear muscle cells (Fig 8).

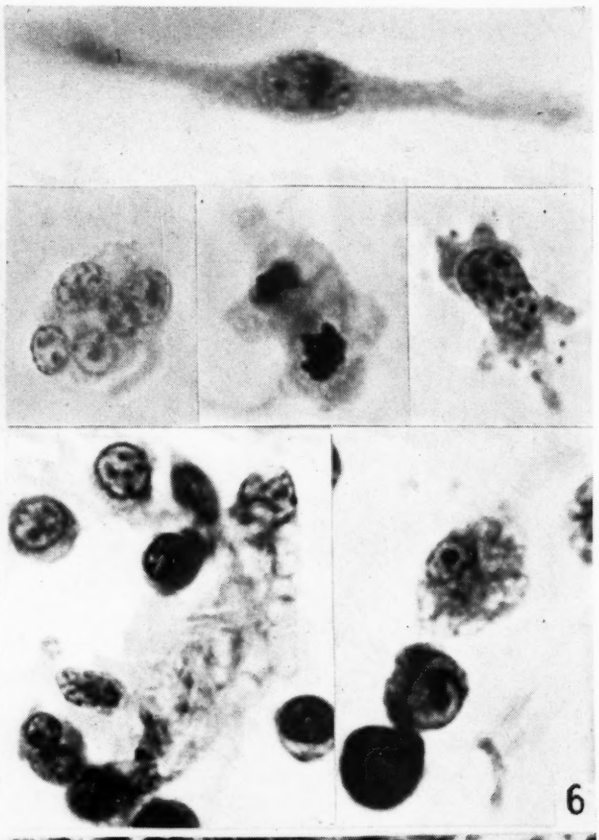
All the tumors at the site of inoculation showed varying combinations of the above described features. In some, the lymphoid cells prevailed; in others, fibroblasts. More or less extensive areas of disintegrating tissue adjoining the newly formed one were often found.

In one exceptional case a big tumor (plum sized) arose in a chicken which died 11 days after injection. It differed from all the other tumors in being a pure hemocytoblastoma, such as mentioned by Engelbreth-Holm. It consisted of densely packed lymphoid cells imbedded in a scarcely visible stroma (Fig. 11). This tissue resembled a young culture of hemocytoblasts (Fig. 12). Though no other instance of a pure hemocytoblastoma was found, densely packed lymphoid cells occurred in other tumors in the vicinity of proliferating spindle cell areas.

## DISCUSSION

Our experiments showed that this peculiar type of local growth was probably not due to the action of the agent. Investigation of the tumors indicates that they may be due to the ability of hemocytoblasts to transform and build a new tissue if given an opportunity to spread and multiply. This was observed by us both *in vitro* and *in vivo*.

The experiments *in vitro* on the cultivation of leukotic blood and bone marrow show that the fate of stem cells was as follows: After a few days of cultivating in hanging drop or Carrel flasks some of the hemocytoblasts perish. Some transform into polyblasts which later transform into fibroblasts, while others transform into fibroblast directly. Some multiply as hemocytoblasts, forming groups and colonies of round cells which are sometimes scattered all over the flasks (Fig. 10).



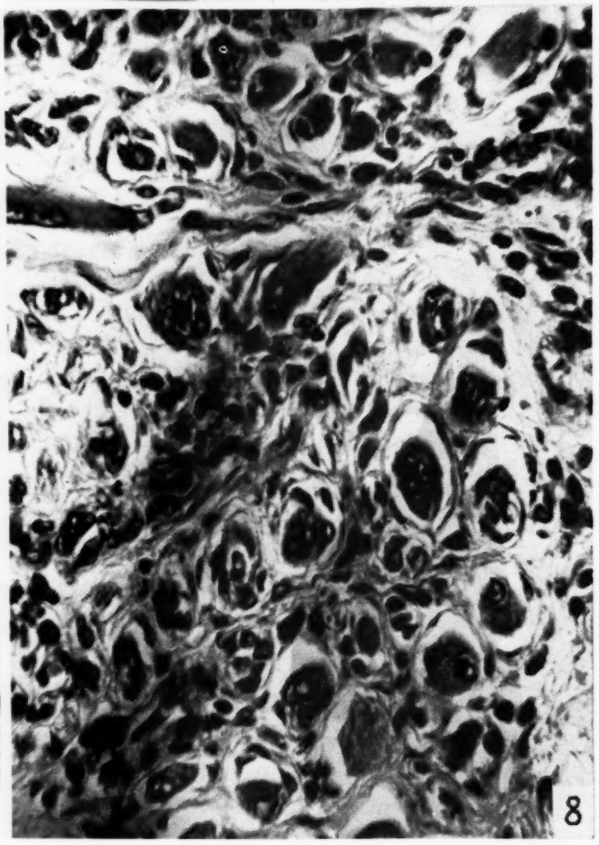
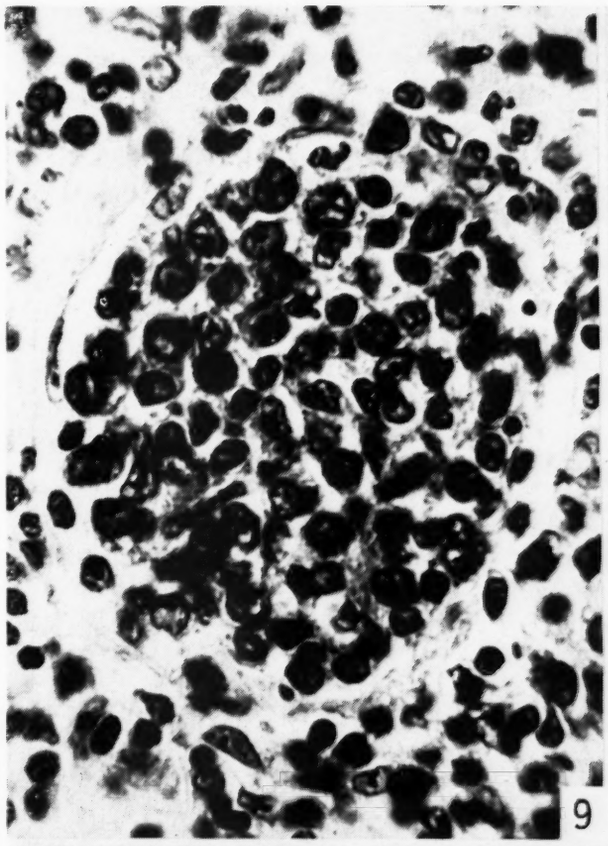


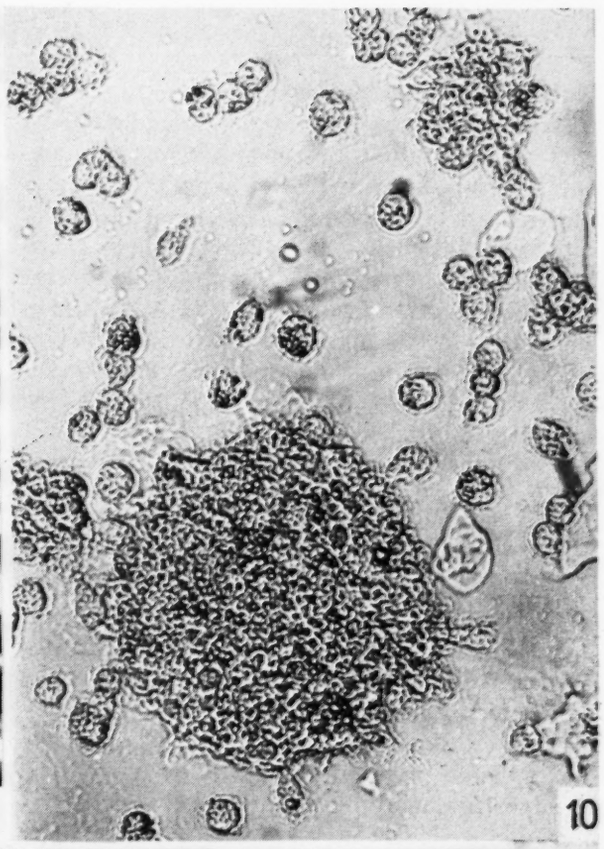
FIG. 5.—Part of a 3 day old culture of leukotic blood cells showing round cells, fibroblasts, and elongated cells. Mag. ×340.

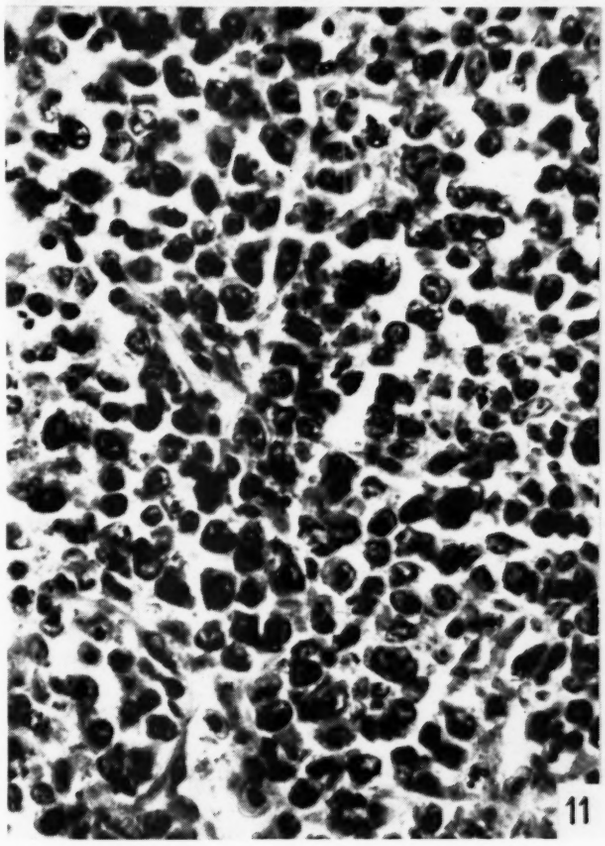
FIG. 6.—Different types of cells resulting from the transformation of stem cells *in vitro*. Mag. ×1340 to 1800.

FIG. 7.—Tissue resembling an angiosarcoma, showing loosely arranged round and elongated cells and cells of endothelial type. Newly formed capillaries are seen. Mag. ×570.

FIG. 8.—Tissue around the nodule represented on figure 9 showing multinuclear muscle cells. Mag. ×520.







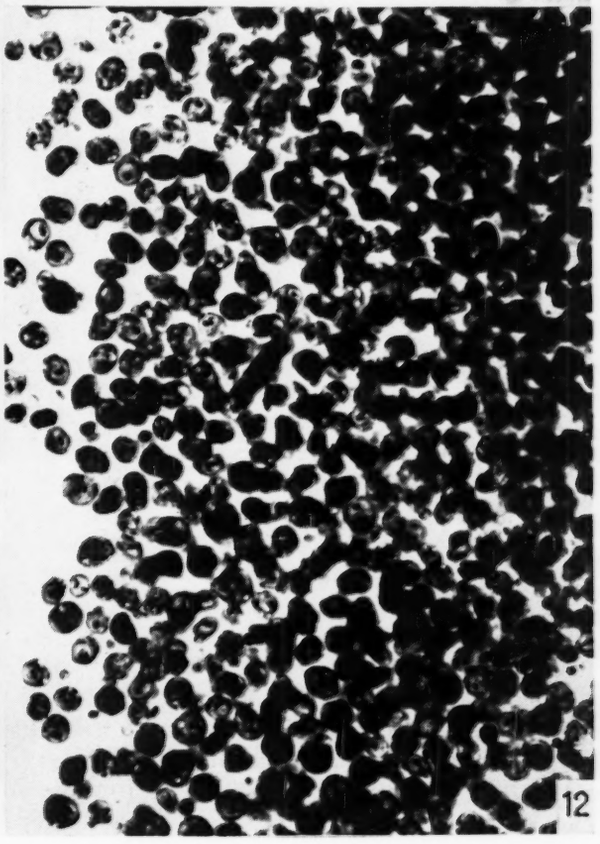


FIG. 9.—A nodule in the muscle tissue in the vicinity of the tumor consisting of lymphoid cells of different size. Between them many hemocytoblasts can be recognized. Mag. ×1040.

FIG. 10.—A colony of hemocytoblasts. Culture in Carrel flask. Mag. ×300.

FIG. 11.—Part of a hemocytoblastoma consisting of densely packed lymphoid cells of different size. Mag. ×660.

FIG. 12.—Hemocytoblasts in a 2 days old culture of blood cells. Mag. ×550.

This behaviour *in vitro* is probably due to the fact that the stem cells in our material were of different degree of maturation and had different developmental potentialities. In Carrel flasks the hemocytoblasts persisted between the proliferating fibroblasts for 2 to 3 weeks, but after one or two passages pure cultures of fibroblasts resulted. The inoculation of such cultures produced leukosis and in 6 out of 18 chickens a tumor arose at the site of inoculation. It is interesting to note that the inoculations of cultures of leukemic agent in normal tissue constantly resulted in leukosis but tumors never appeared at the site of inoculation (5).

Study of the outer and inner zones of leukotic tumors in different stages of their development shows that in the first days following inoculation of leukotic material the hemocytoblasts spread into the muscle tissue. Some of them degenerate and decompose. The surviving ones multiply actively between the muscle fibres and finally begin to transform. As in tissue culture, the stem cells transform either directly into fibroblasts or pass through the stage of a polyblast. The fibroblasts themselves multiply, build the inner part of the tumor, and sometimes invade the tissue together with the hemocytoblasts or alone. Some of the hemocytoblasts which penetrate between the muscle fibres multiply as such, as they also do *in vitro*, and form nests of lymphoid cells of different size. In this way the characteristic features of the leukemic tumors are built.

The experiments *in vitro* and *in vivo* seem to throw some light on the problem of tumor formation following inoculation of leukotic material of different leukemic strains. In the first part of this investigation we discussed the difference between the combined and pure leukotic strains. It appears that the intramuscular inoculation of cell-free agent of combined strains such as strain 13 of Stubbs and Furth (6) and strain ES of Engelbreth-Holm (7, 8) can produce transferable malignant metastasizing tumors. The agents of pure leukosis strains either do not produce tumors at all or can produce only a local growth after inoculation of cell-containing material. Such growth does not spread, is not transmissible, does not metastasize, and cannot be considered as malignant. The pure strain of Jarmai and strain T of Engelbreth-Holm can produce a local growth after intramuscular inoculation of cell-containing material. The strain I of Furth (9) never produced tumor by intramuscular inoculation. This difference can be explained on the basis of our experiments. The strain I of Furth produces erythroblastosis-myelosis. The primitive cells which dominate in this strain are erythroblasts and myeloblasts. These cells are more mature and differentiated than the hemocytoblasts. They cannot transform into fibroblasts. They can either mature to normal blood cells or perish. This virus as described by Furth and Breedis (12) can exist in tissue culture only as long as such cells, "presumably myeloblasts," are alive. As they never transform into fibroblasts, the cells soon perish as well as does the virus. The same thing happens when such cells are injected intramuscularly. Therefore, no growth at the site of inoculation can take place. On the other hand when leukotic material of strain T of Engelbreth-Holm or of the strain of hemocytoblastosis of Jarmai, containing stem cells, is injected intramuscularly a tumor develops as a result of transformation and multiplication of these cells.

There is probably a fundamental difference between the histogenesis of tumors produced by combined and pure leukotic strains. Whereas the first may be the result of virus action on connective tissue the second are dependent on the potentiality of the inoculated cells.

This may sometimes also be the cause of tumors in the combined strains. Rothe Meyer and Engelbreth-Holm in the course of their study of the combined strain ES (7) observed polygonal cells which infiltrate the surrounding tissue around hemorrhages in the liver, spleen, and bone marrow. All transition forms from such cells to fibroblasts were seen in such areas. The process resulted in angiosarcomatous infiltration. The same kind of tissue "eine intime Mischung von fusozellulaerem, endotheliomatosem und angiosarkomatosem Gewebe" were seen after inoculation of blood of this strain, whereas transplantation of tumor of the same strain resulted in fibrosarcoma. The same authors never observed transition forms with strain T. They supposed that a more immature cell (hemo-histioblast) was involved in strain ES than in strain T and R. They discussed the following possibilities of tumor formation by material from pure strains: a) from the injected hemocytoblasts which become converted into malignant fibroblast; b) from the tissue of the host as a result of the effect of the leukotic agent; c) both possibilities co-exist. The first and third possibilities were excluded as transition forms were not observed after inoculation of blood of strain T and were considered as highly improbable. The second possibility was supposed to be more probable because the inoculation of plasma resulted in tumor in one case with strain T and in two cases with strain R. The conspicuous difference in results of inoculation of plasma and blood was explained by

differences in concentration of the leukotic agent. In our experiments with strain T we saw all transition forms from hemocytoblasts to fibroblasts in tissue culture and after intramuscular inoculations. There was no difference in concentration of the agent in the irradiated and non-irradiated liver; nevertheless the first never produced tumors whereas the second regularly produced tumors. We do not know how to explain the difference between our experiments and those of Engelbreth-Holm, but we feel more inclined to suppose that the growth resulting at the site of inoculation of cell-containing material from the "pure" strain T is the result of the potentialities of the hemocytoblasts present in the inoculated material, whereas in combined strains the same process can also happen as a result of hemorrhage or inoculation of blood. Apparently the cell-free agents of the pure leukotic strains have affinity for primitive blood cells only and wherever inoculated produce leukemia, just as the Rous sarcoma agent has affinity for connective tissue cells only and always produces sarcoma. Agents of combined strains can probably act on both bone-marrow cells and fibroblasts of the connective tissue, producing leukemia by intravenous inoculation and sarcoma by intramuscular application.

## SUMMARY

1. As was shown in the previous paper, cell-free leukotic material never produced a tumor at the site of inoculation, whereas the same material containing live hemocytoblasts regularly induced a tumor at the site of inoculation.

2. A description of different parts of such tumors is given and a parallel is drawn between the fate of stem cells *in vitro* and *in vivo*.

3. A discussion on the histogenesis of tumors produced by "pure" leukotic strains points to the supposition that such tumors are probably a result of multiplication and transformation of stem cells present in the inoculum.

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# The Effect of Bromobenzene and 3,4-Benzpyrene on the Metabolism of Radioactive L-Cystine*†

HELMUT R. GUTMANN AND JOHN L. WOOD

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The detoxication of aromatic hydrocarbons and their halogen derivatives has been studied for many years. It has been established that these compounds undergo the biochemical reactions of oxidation to dihydrodihydroxy compounds, phenols, and quinones; of oxidative ring scission to form carboxylic acids; and of conjugation with cysteine to form mercapturic acids.[1] With the discovery of the carcinogenic activity of certain polycyclic aromatic hydrocarbons, interest in the metabolism of aromatic hydrocarbons has been further stimulated. Some of the predictable detoxication products such as the phenols and quinones of 3,4-benzpyrene (2, 3) and 1,2,5,6-dibenzanthracene (1) have been isolated. Recently, Heidelberger, Kirk, and Perkins (11) have demonstrated the oxidative fission of some of the rings of dibenzanthracene.

The isolation of mercapturic acids from administered carcinogenic hydrocarbons has never been accomplished. Nevertheless, the concept of the participation of sulfur amino acids in the metabolism of carcinogenic hydrocarbons has persisted. Haddow, Scott, and Scott (9) showed that 1,2,5,6-dibenzanthracene, 1,2,5,6-dibenzacridine, and 3,4-benzpyrene produced immediate and long-continued reduction in the growth of rats. White and White (14) found that 20-methylcholanthrene and benzpyrene produced an inhibition of growth in rats and suggested that, similar to bromobenzene, these compounds were causing a cystine deficiency through mercapturic acid formation. Crabtree (4) found no change in the neutral sulfur of the urines of mice painted with benzpyrene or dibenzanthracene. He concluded that mercapturic acids accounted for no major amounts of the elimination products of these hydrocarbons. However, since nonspecific disturbances in sulfur metabolism reduced the rate of tumor induction by the carcinogens, he retained the hypothesis that a primary action of carcinogens is their combination with sulfhydryl groups in the cell. This point of view was reiterated by Elson, Goulden, and Warren (5), who studied the sulfur partition in urine following the administration of polycyclic hydrocarbons. They found no increase in neutral sulfur corresponding to mercapturic acid formation after the administration of 3,4-benzpyrene or 1,2,5,6-dibenzanthracene, although profound inhibition of growth occurred. Sulfur-containing proteins in excess of the amount needed to permit mercapturic acid formation were fed. With diets containing 20 per cent protein, there was some protection against growth inhibition, but it was not necessarily provided by sulfur amino acids (6, 7). Addition of cystine to a 5 or 10 per cent casein diet failed to prevent growth inhibition. The action of the two hydrocarbons, therefore, could not be attributed to a simple deprivation of the organism's requirements for cystine and methionine, such as occurs with growth inhibition by bromobenzene and anthracene. The authors concluded that no direct relationship between sulfur metabolism and growth inhibition was detectable within the limits of sensitivity of their method. This did not preclude the possibility of a combination of small amounts of the hydrocarbon with sulfhydryl-containing substances or the possibility that the breakdown of such combinations by subsequent metabolic action might not lead to some mercapturic acid excretion.

The formation of mercapturic acids from administered aromatic hydrocarbons as a general metabolic reaction was still an open question at the time the present investigation was started. If the carcinogens give rise to mercapturic acids, the amounts excreted must be very small. Because the amount of carcinogen required to initiate tumor formation is also very small, more sensitive methods were desirable for the determination of mer-

* This work was supported by a grant from the American Cancer Society upon recommendation by the Committee on Growth of the National Research Council.

† A preliminary report of this work was presented to the American Society of Biological Chemists, Detroit, April 21, 1949.

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[1] For a review of the metabolism of aromatic hydrocarbons, see Williams, R. T., Detoxication Mechanisms. New York: Wiley & Sons, Inc., 1947.

capturic acid formation than those previously used. The use of radioactive sulfur afforded an accurate method for this purpose.

In the first experiments, L-cystine was prepared by the method of synthesis and resolution previously described (15, 17). The compound was administered to rats by intraperitoneal injection. Some of the rats were given bromobenzene, as an example of a compound known to form a mercapturic acid; and others were given 3,4-benzpyrene. The urines were studied for the presence of mercapturic acids and for changes in the sulfur partition. The specific radioactivity of the sulfur used permitted the detection of as little as 0.2 micrograms of mercapturic acid sulfur, arising from the injected cystine and found in a chloroform extract of the urine. The results in Table 1 show that the expected amount of mercapturic acid (12) was found in the urine after a feeding of bromobenzene. After administration of a large dose of 3,4-benzpyrene, however, no significant amount of radioactive sulfur was found in the chloroform extract. Thus it appears that cystine, administered simultaneously with benzpyrene, forms no mercapturic acid within 48 hours.

TABLE 1

EFFECT OF INJECTED HYDROCARBONS ON THE URINARY PARTITION OF $S^{35}$ FOLLOWING THE ADMINISTRATION OF LABELED L-CYSTINE

| Rat | L-cystine given (mg.) | Hydrocarbon administered (mg.) | Days | Total labeled S excreted (μg.) | Total labeled $SO_4$-S (μg.) | Labeled $CHCl_3$-S (μg.) | Total $CHCl_3$-S (mg.) | Total mercapturic acid excreted (mg.) |
|---|---|---|---|---|---|---|---|---|
| A | 10* | None | 1 | 470 | 379 | | | |
| | | | 2 | 92 | | | | |
| | | | 3–6 | 266 | | | | |
| B | 10.7† | None | 1–2 | 490 | 360 | 6.7 | | |
| | | | 3–4 | 113 | | | | |
| C | 11.4† | None | 1–2 | 412 | 200 | 0.7 | | |
| D | 10* | 150 mg. bromobenzene | 1 | 342 | 272 | 123 | 11.7 | 116 |
| | | | 2 | § | | | | |
| | | | 3–6 | 94 | | | | |
| E | 10* | 150 mg. bromobenzene | 1 | 420 | 296 | 101 | 10.3 | 103 |
| | | 150 mg. bromobenzene | 2 | § | | | | |
| | | 150 mg. bromobenzene | 3–6 | 142 | | | | |
| F | 9.7‡ | 30 mg. bromobenzene | 1 | 355 | 256 | 32 | | |
| G | 10.2† | 37 mg. benzpyrene | 1–2 | 390 | 270 | 0.6 | | |
| | | | 3–4 | 380 | | | | |
| H | 10.0† | 63 mg. benzpyrene | 1–2 | 420 | 347 | 0.6 | | |
| | | | 3–4 | 107 | | | | |

* Specific activity, 0.27 counts/sec/μg. S.
† Specific activity, 1.84 counts/sec/μg. S.
‡ Specific activity, 0.133 counts/sec/μg. S.
§ The radioactivities of these samples exceeded the background activity by less than 10 per cent.

## MATERIALS AND METHODS

*Animals.*—Male rats weighing from 200–300 gm. were set up in individual glass metabolism cages which allowed the separation of urine and feces. Urine and feces were collected for the periods of time stated in the tables. The urine was preserved under toluene with the addition of a few drops of concentrated hydrochloric acid.

*Diet.*—The rats were maintained on a diet of the following composition: sucrose 23 per cent, starch 23, casein 17, salt mixture (10) 4, corn oil 3, lard 10, cod liver oil 2, dry yeast 14, celluflour 4. Water and solid food were given ad lib.

*Radioactive cystine.*—Labeled L-cystine was prepared by the methods previously described (15, 17). The radioactive compound was dissolved in a small volume of N HCl and the solution concentrated *in vacuo* to about 0.2–0.5 ml. Approximately 2 ml. of warm N $NaHCO_3$ were added, and the resulting solution was injected intraperitoneally.

*Aromatic hydrocarbons.*—The aromatic hydrocarbons were administered by stomach tube in the amounts stated in the tables. Large quantities were used to favor absorption of significant amounts from the gut. Prior to administration, bromobenzene was redistilled and the fraction, boiling at 42°–44.5°, was employed. The melting point of the 3,4-benzpyrene[2] was 178°–179° (corr.). For administration, 3,4-benzpyrene was dissolved in warm corn oil.

*Urinary sulfur partition.*—The distribution of the radioactive sulfur in the urine was measured as follows: The urine was filtered through glass wool and diluted to a convenient volume (usually 25 ml.) with distilled water. The inorganic phosphate

[2] We are indebted to Dr. L. F. Fieser for the 3,4-benzpyrene used in this study.

was removed as previously described (8). The total radioactive sulfur of an aliquot of the urine was converted to sulfate as described below. The sulfate was precipitated as benzidine sulfate, and the radioactivity of the precipitate was measured (16).

Another suitable aliquot of the urine was acidified to Congo Red and was extracted twice with 10 ml. of chloroform and once with 5 ml. of the same solvent. The chloroform extract was centrifuged and the supernatant aqueous layer returned to the separatory funnel. The chloroform extract was washed once with 5 ml. of distilled water, and the wash liquid was discarded. The chloroform extract was filtered, and the solvent was removed by evaporation. The dry residue was combusted, and the sulfate was precipitated as benzidine sulfate. The radioactivity of the precipitate was measured as described below.

Total inorganic sulfate sulfur was estimated on a suitable aliquot of the urine, after preliminary chloroform extraction. An appropriate volume of distilled water was added to the aliquot, and the solution was heated under reflux for 5–10 minutes. The solution was then concentrated at atmospheric pressure to about 10 ml. and cooled. The inorganic sulfate was precipitated by the benzidine reagent, and the radioactivity of the precipitate was measured.

The total sulfur of the urine and the sulfur extractable from the urine with chloroform were each converted to sulfate, in the majority of cases by the semimicro-Carius procedure, and in a few instances by alkaline fusion, or by wet oxidation with perchloric acid. All measurements were carried out in duplicate.

*Radioactive fecal sulfur.*—An air-dried aliquot of the feces was combusted with perchloric acid after preliminary digestion with 1–2 ml. of concentrated nitric acid. The digest was made up to 10–15 ml. with distilled water, and the inorganic phosphate was removed. Sulfate was precipitated from a suitable aliquot of this solution with benzidine, and the radioactivity of the precipitate was measured.

*Radioactivity measurements.*—The radioactivities of the various samples were measured with a thin-window type Geiger-Müller counter. Correction for self-absorption by the sample was applied. A radioactive standard was prepared from the same radioactive cystine preparation which supplied the sample used in the metabolic studies. By comparison with the standard sample, the radioactivities of the various samples obtained from the fractionation of the urine were expressed as micrograms of radioactive sulfur.

*Isolation of radioactive bromophenylmercapturic acid from rat urine.*—Two male rats each received 9.7 mg. of radioactive L-cystine intraperitoneally. One was given 15 mg. and the other 30 mg. of redistilled bromobenzene by stomach tube. The urine was collected for 24 hours and filtered; 100 mg. of unlabeled bromophenylmercapturic acid were dissolved in warm, dilute ammonium hydroxide and added to 60 ml. of the filtered urine. The urine was acidified to Congo Red with concentrated hydrochloric acid and extracted twice with 15-ml. portions of chloroform. The chloroform extracts were combined and evaporated under reduced pressure. The dry residue was dissolved in the minimum amount of dilute ammonium hydroxide and precipitated by the addition of hydrochloric acid to Congo Red. The precipitate was filtered with suction and washed with cold distilled water and ether. A fraction of the material (m.p. 150°–153° corr.) was combusted after drying over phosphorus pentoxide *in vacuo* for 12 hours. The sulfate was precipitated as benzidine sulfate, and the radioactivity of the precipitate was measured. The remaining material was recrystallized again from a mixture of absolute alcohol and dilute hydrochloric acid. After standing in the refrigerator for 2 days, the precipitate was collected, washed, and dried as described above. The melting point of the material was 155° (corr.). After oxidation of the sample by alkaline fusion and precipitation of the sulfate by benzidine, the radioactivity of the benzidine sulfate was redetermined.

## RESULTS AND DISCUSSION

The distribution of radioactive sulfur in the urine of the rat after the injection of labeled L-cystine and the effect of bromobenzene on the partition of the radioactive sulfur are shown in Table 1. Approximately 16 per cent of the administered radioactive sulfur was excreted in the urine within 48 hours. The chloroform extract of the urine contained significant amounts of radioactive sulfur only after administration of bromobenzene. A remarkable absence of radioactivity in the urine of Rats D and E was observed the second day after bromobenzene.

The incorporation of the radioactive sulfur into bromophenylmercapturic acid was confirmed by the addition of unlabeled bromophenylmercapturic acid to the chloroform extract and subsequent isolation of radioactive bromophenylmercapturic acid. The specific radioactivity of the isolated material remained constant with successive recrystallizations of the radioactive compound from two different solvents.

The amount of chloroform-extractable sulfur depended upon the dose of bromobenzene. This

quantity, however, represented only 1–4 per cent of the administered radioactive cystine sulfur and accounted only for a small fraction of the excreted bromophenylmercapturic acid (Table 1). It may be calculated from gravimetric determinations of the sulfur in the chloroform extracts that a total of 116 and 113 mg. of bromophenylmercapturic acid was excreted by Rats D and E, respectively. These values are in good agreement with data obtained by Stekol (12) for the excretion of bromophenylmercapturic acid by the rat. It is apparent from the data of Table 1 that considerable dilution of the radioactive sulfur by unlabeled sulfur had occurred. This dilution arose from two sources: the sulfur of the diet eaten by the rats and the tissue sulfur compounds. The food eaten by each rat in 24 hours furnished approximately 21 mg. of sulfur in the form of cystine and methionine, while the injected cystine furnished 2.7 mg. Thus a total of 23.7 mg. of exogenous sulfur was available for mercapturic acid formation during the 24-hour period. It may be calculated from the data of Table 1 that 4.6 and 3.8 per cent of the administered radioactive sulfur were converted to mercapturic acid. If it can be assumed that the injected and ingested sulfur became homogeneously mixed in the rat, then approximately 4 per cent of the 23.7 mg. of exogenous sulfur was used for mercapturic acid formation. This amount of sulfur (0.95 mg.) represents less than 10 per cent of the total mercapturic acid produced by the rat in 24 hours.

These data thus give independent confirmation to the conclusion reached by Stekol (13) that the greater part of the sulfur of bromophenylmercapturic acid must be derived from nondietary compounds.

The effect of 3,4-benzpyrene on the sulfur metabolism of the rat is illustrated in Table 1. A large dosage was administered in order to obtain maximal absorption of the compound from the gut. It will be seen that the distribution of the radioactive sulfur in the urine fails to show any significant differences between the controls and the rats which had received 3,4-benzpyrene. The radioactivity of the chloroform-extractable sulfur fraction slightly exceeded the background radioactivity in all cases, suggesting the presence of minute amounts of chloroform-extractable radioactive sulfur in the urine. This doubtless arose from the lack of complete immiscibility between chloroform and water and the high specific radioactivity of the labeled sulfur used. It thus represents a limitation of the sensitivity of the determination of radioactive mercapturic acid by extraction.

The lack of interference of 3,4-benzpyrene with the over-all sulfur metabolism of the rat is also indicated by the data of Table 2. The fraction of the radioactive cystine sulfur which was recovered during four days from the urine and the feces of two control animals, Rats B and C, and from two animals which had received 3,4-benzpyrene, Rats G and H, were almost identical.

## SUMMARY

The effects of bromobenzene and 3,4-benzpyrene on the sulfur metabolism of the rat have been investigated with the aid of L-cystine labeled with radioactive sulfur. Isolation of radioactive bromophenylmercapturic acid from the urine of rats which had received bromobenzene showed that radioactive sulfur administered as L-cystine was utilized for the synthesis of mercapturic acid. About 4 per cent of the labeling sulfur appeared in the mercapturic acid fraction within 24 hours after the simultaneous administration of bromobenzene and L-cystine. Less than 10 per cent of the total mercapturic acid excreted in 24 hours came from exogenous sulfur.

The administration of 3,4-benzpyrene produced no effect on the sulfur metabolism of cystine which was injected simultaneously with the hydrocarbon. In particular, there was no evidence for the formation of a mercapturic acid resulting from the conjugation of 3,4-benzpyrene with cystine.

TABLE 2

RECOVERY OF URINARY AND FECAL RADIOACTIVE SULFUR DURING 4 DAYS FOLLOWING THE ADMINISTRATION OF RADIOACTIVE L-CYSTINE AND 3,4-BENZPYRENE*

| Rat | Total urinary sulfur (μg.) | Total fecal sulfur (μg.) | Total sulfur (μg.) | Per cent of the administered dose |
|---|---|---|---|---|
| B | 603 | 38 | 641 | 23 |
| C | 509 | 167 | 676 | 22 |
| G | 470 | 112 | 582 | 21 |
| H | 527 | 115 | 642 | 24 |

* Rat B received 10.7 mg. of radioactive L-cystine.
Rat C received 11.4 mg. of radioactive L-cystine.
Rat G received 10.2 mg. of radioactive L-cystine and 37 mg. of 3,4-benzpyrene.
Rat H received 10.0 mg. of radioactive L-cystine and 63 mg. of 3,4-benzpyrene.

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# Studies on the Promoting Phase in the Stages of Carcinogenesis in Mice, Rats, Rabbits, and Guinea Pigs

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In previous experiments by Berenblum and Shubik (4), tumors were induced in mice by a single application of a carcinogenic hydrocarbon, followed by repeated applications of noncarcinogenic croton oil. Friedewald and Rous (7) similarly showed that tumors may be induced in the rabbit by a suboptimal dose of carcinogen followed by wound healing and applications of nonspecific agents, such as turpentine. Previously, Berenblum (1) had shown that turpentine, as well as croton oil, had an augmenting (cocarcinogenic) effect in carcinogenesis in mice; and several other examples of this activity have been reviewed in the literature by Berenblum in 1944 and Rusch (15). Arising from this work, a new concept of carcinogenesis has been proposed, involving an initiating stage, brought about by the hydrocarbon, with a small number of normal cells changing into latent tumor cells which are subsequently converted into morphological tumors by promoting action (e.g., croton oil, wound healing, etc.). The latent tumor cells remain irreversibly changed for 20 weeks (4) or even as long as 43 weeks (5).

In the mouse, only croton oil can be said to have considerable promoting action, whereas other factors previously investigated, such as turpentine (1) and wound healing (6, 13) have a minimal effect. In the rabbit, on the other hand, both wound healing and turpentine seem to have a marked effect.

Substances tested for possible promoting activity have usually been selected from those agents known to induce epidermal hyperplasia. Mice and rabbits have most frequently been employed for such tests. In the present study, a variety of substances, all inducing epidermal hyperplasia, was examined for possible promoting activity, and, in addition to mice and rabbits, rats and guinea pigs were also used.

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## METHODS

The mice used in this investigation were of the Swiss strain from the Medical Research Council, England, and were bred in this laboratory. Rabbits, rats, and guinea pigs were from the stock of the same laboratory. Throughout the investigation 9,10-dimethyl-1,2-benzanthracene as a 1.5 per cent solution in medicinal liquid paraffin (light mineral oil) was used as the initiating agent. Liquid paraffin was used as the solvent, except where solubilities made this impracticable. Test solutions were applied with a fine glass rod.

## EXPERIMENTAL

Mice.— In this series, turpentine applications, already shown to have a mild cocarcinogenic effect in mice (1) and a noticeable action in rabbits (7), were reinvestigated as promoting agents after a single application of 1.5 per cent 9,10-dimethyl-1,2-benzanthracene. Next, a series of substances was examined in a similar way: (*a*) phenanthrene and fluorene, which are noncarcinogenic in mice (9, 10); (*b*) acridine, investigated by Kennaway (9) and Maisin *et al.* (11), and found to be noncarcinogenic but very irritating; (*c*) a series of vegetable oils, e.g., castor oil, its active principle ricinoleic acid, glyceryl monoricinoleate, and oleic acid, which had been shown (17) to augment carcinogenesis with inadequate dosage of 3,4-benzpyrene; (*d*) silver nitrate in aqueous solution; and (*e*) cauterization by electrocautery.

Each of these substances was first applied in serial dilutions to a small area of normal skin, in the interscapular region, which was clipped free of hair with scissors. After four applications, spaced over 2 weeks, the mice were killed, and pieces of tissue removed for histologic examination. The concentration yielding the maximum hyperplasia with the minimum of necrotic change was chosen for further testing. For the promotion tests, groups of ten mice were used, each receiving a single application of the carcinogen, and, after an interval

of 3 weeks, twice-weekly applications of the test solution. The turpentine was used as a 20 per cent solution, phenanthrene as a 1 per cent, acridine as a 0.3 per cent, and fluorene as a 0.5 per cent solution, all in liquid paraffin. Silver nitrate was made up as a 10 per cent aqueous solution. The remaining test substances were used undiluted. Finally, a group of five mice was cauterized with a superficial linear streak 1 inch long in the center of the painted area at monthly intervals following a single application of the carcinogen. In this group no tumors have been recorded at 60 weeks following the original application.

The results of the tests for promoting activity, summarized in Table 1, were uniformly negative.

TABLE 1

SUBSTANCES TESTED FOR TUMOR PROMOTION IN MICE

| Substance tested | Skin histology* | Promotion test† |
|---|---|---|
| 20 per cent Turpentine in liquid paraffin | Minimal histologic change | No tumors recorded |
| 0.3 per cent Acridine in liquid paraffin | Marked epidermal hyperplasia | " |
| 0.5 per cent Fluorene in liquid paraffin | Slight epidermal hyperplasia | " |
| 1 per cent Phenanthrene in liquid paraffin | " | " |
| Castor oil | " | " |
| Ricinoleic acid | " | " |
| Glyceryl monoricinoleate | " | " |
| Oleic acid | " | " |
| Silver nitrate (10 per cent aqueous) | Good hyperplasia, most marked in the hair follicles | " |

* Groups of three mice; 4½ weekly applications.

† Groups of ten mice, given semiweekly applications for 20 weeks, 3 weeks after a single application of 9, 10-dimethyl-1,2-benzanthracene in liquid paraffin.

RABBITS.—The experiments of Friedewald and Rous (7), showing that wound healing may act as a promoting agent in this species, were repeated but modified by using *only a single application* of carcinogen as initiator. Turpentine has been reinvestigated under these conditions, and the effects of croton oil studied in the rabbit. Similarly, chloroform and inorganic arsenic were reinvestigated under these conditions.

Berenblum (2) showed that 9,10-dimethyl-1,2-benzanthracene is a potent carcinogen for rabbit skin, and a single application of this hydrocarbon was therefore used as standard initiator for this part of the investigation also.

*The effects of wound healing and turpentine.*—Four areas of skin on the back of one rabbit, clipped free of hair with electric clippers, were used for the determination of the optimal dosage of turpentine; four strengths, undiluted, and 50, 20, and 10 per cent turpentine in liquid paraffin, were applied to these areas twice weekly. The undiluted turpentine induced ulceration within 1 week, and it was discontinued. The other three solutions induced macroscopic reddening and thickening of the skin. After 3 weeks the 50 per cent solution also caused some ulceration, and it, too, was discontinued; a skin biopsy was made for histological examination. The remaining concentrations were applied for up to 10 weeks, and the animal then killed. Histologically, extensive epidermal damage was noted in the skin treated with 50 per cent solution, and only minimal hyperplasia; with the 20 per cent solution there was considerably less damage, but, again, only minimal hyperplasia. The 10 per cent solution induced almost no change. In no case was an appearance produced resembling that described by Rous (14), where turpentine caused the epidermis to become from five to ten cells thick.

In spite of the unsatisfactory nature of the first test, but because of earlier favorable reports, the experiment was continued, using the 20 per cent solution, applied to three areas of skin on the backs of four rabbits, which had been treated 1 month previously with a single application of the carcinogen. The fourth area on each rabbit was maintained as a control for one application of carcinogen. These applications were continued twice weekly for 20 weeks; one rabbit died in the sixth week of the experiment and yielded no results. Concurrently, a hole ¾ inch in diameter was punched through the ears of each rabbit previously treated only once with carcinogen, using a sharp cork borer. Among the three surviving rabbits, one developed definite papillomas on both ears at the seventh week around the site of the punched hole, and another developed a papilloma on one ear at the eighth week following the trauma. All of these tumors continued to grow until the twentieth week when the animals were killed, but none became malignant. On the third rabbit tumors did not develop. There was no evidence of any tumor formation in any of the areas treated with turpentine.

*The effects of croton oil on rabbit skin.*—Again preliminary skin tests were carried out on one rabbit, with a 5 per cent solution of croton oil applied to two areas of skin and a 10 per cent solution to two others, at twice-weekly intervals for 2 weeks. Both solutions were made up in liquid paraffin. The skin showed some macroscopic reddening and thickening following the 5 per cent solution, and ulceration following the 10 per cent. Histologically it was noted that both solutions induced some epidermal necrosis, far more pronounced with the stronger solution; and in both cases there was

patchy epidermal hyperplasia. Four areas of skin on three rabbits were then treated with a single application of the carcinogen, and, after an interval of 1 month, applications twice weekly of croton oil (5 per cent) were begun. No tumors were recorded after 20 weeks.

*The effects of arsenic and chloroform.*—Arsenic trioxide and arsenic pentoxide were each made up as a 5 per cent preparation in a Lanette wax SX ointment base. The chloroform was applied undiluted. Four areas of skin of two rabbits were clipped free of hair, and to three of these was applied a single application of the carcinogen. After an interval of 1 month, the three areas were treated with one or another of the test compounds. The areas not treated with carcinogen were used as controls for the effects of the arsenic alone. The experiment was concluded at 20 weeks from the commencement of the second series of applications. No tumors were recorded.

GUINEA PIGS.—

*The effects of croton oil.*—Studies on tumor formation with the guinea pig have been few, although Haagensen and Krebhiel (8) and Shimkin and Mider (16) have shown that the induction of sarcomas, using 3,4-benzpyrene and 20-methylcholanthrene, is possible within limits comparable to those occurring among other laboratory animals. Recently Berenblum (5) has shown that 9,10-dimethyl-1,2-benzanthracene is an effective carcinogen for guinea-pig skin, although the average latent period is as long as 51 weeks.

Preliminary skin tests on normal guinea pigs were carried out with 5 and 10 per cent solutions of croton oil in liquid paraffin. The 5 per cent solution induced considerable hyperplasia with minimal damage. Solutions were applied to both flanks of the animals, which were clipped free of hair with electric clippers. The effects of a limited number of applications of the carcinogen, followed by repeated applications of croton oil, were then investigated. Four groups of five guinea pigs were used for this:

*Group 1.*—both flanks were given three applications of carcinogen at twice-weekly intervals; after a 4-day interval only the left flanks received, twice weekly, 5 per cent croton oil applications.

*Group 2.*—the left flanks only were given three applications of the carcinogen; after a 4-day interval both flanks received twice-weekly applications of croton oil.

*Group 3.*—both flanks were given three applications of carcinogen; after a 4-day interval both received croton oil applications.

*Group 4.*—both flanks received but a single application of carcinogen, followed by the croton oil.

The experiment was continued for 33 weeks from the commencement of the croton oil applications, and no tumors were recorded. However, by the end of the experiment the animals were all in bad condition, having scratched the treated areas considerably. The procedure was then repeated on another group of 30 guinea pigs, divided into three groups of ten; only a single area of skin, in the interscapular region, was used for the applications in each animal. One group received ten applications of carcinogen, and another five, at twice-weekly intervals, as it was considered that the three applications used originally might have been insufficient in this species. The third group received croton oil only. The experiment was continued for 36 weeks, but again no tumors were induced, and the skins of the animals were found to be in bad condition; a sensitivity to croton oil apparently developed after a few applications in some guinea pigs, which necessitated periodic cessation of treatment to prevent frank ulceration.

TABLE 2

THE EFFECT OF IRRITANTS ON THE SKIN OF ANIMALS

| Species | Agent tested | Skin histology* | Promotion test† |
|---|---|---|---|
| Rabbits | Wound healing‡ | | Several tumors induced |
| | 20 per cent turpentine in liquid paraffin | Slight epidermal hyperplasia | No tumors induced |
| | 5 per cent croton oil in liquid paraffin | " | " |
| | Chloroform | " | " |
| | 5 per cent arsenic trioxide in Lanette wax SX | " | " |
| | 5 per cent arsenic pentoxide in Lanette wax SX | " | " |
| Rats | 5 per cent croton oil in liquid paraffin | Some atrophic changes in dermis | " |
| Guinea pigs | 5 per cent croton oil in liquid paraffin | Marked epidermal hyperplasia | " |

* On normal skin after 4½ weekly applications of the agents.

† On skin painted with a single application of 9,10-dimethyl-1,2-benzanthracene (or more: see text), and then treated with the test agent twice weekly.

‡ Performed by punching a hole, ¾ inch in diameter, through the ear with a sharp cork borer.

RATS.—

*The effects of croton oil.*—Studies on tumor formation of the skin of rats are more numerous than those with guinea pigs, and recently Berenblum (5) has shown that 9,10-dimethyl-1,2-benzanthracene

is an effective carcinogen for the skin of this species, although the average latent period is 30 weeks. Preliminary skin tests with croton oil induced macroscopic thickening and reddening; and an eczematoid lesion of the skin occurred with the 5 and 10 per cent solutions, more marked with the stronger. After four applications of croton oil given over 2 weeks some atrophic changes of the dermis were noted histologically, with minimal damage to the epidermis. In spite of this finding, twenty rats were each given three applications of the carcinogen at twice-weekly intervals, followed after 4 days by twice-weekly applications of 5 per cent croton oil. Another group of ten rats was given the croton oil only. This experiment was complicated by the fact that eczematoid skin lesions arose in all of the animals treated with croton oil. No tumors were recorded at 30 weeks from the commencement of the croton oil treatment.

## DISCUSSION

Under the conditions of these experiments, croton oil possesses a definite specificity for the mouse, for which it is the most potent promoting agent yet investigated. The other substances tested for promoting activity in the mouse all proved negative following a single application of the carcinogen, although some of them had been shown earlier to have a co carcinogenic effect, in combination with repeated applications of carcinogenic hydrocarbons. It is possible that turpentine, for example, is a weak promoting agent, whose activity can only be revealed after more potent initiation.

In the rabbit, wound healing has proved to be by far the most effective method of promoting tumors, although it is likely that the turpentine sample used in this experiment differed in properties from that used by Rous and his collaborators. Inevitably, the continued use of such complex and uncontrolled organic mixtures will make the comparison of results haphazard. However, samples of croton oil, which are quite effective in the mouse, prove completely inactive in the rabbit. Chloroform, too, seems only a very weak promoting agent, requiring more than a single application of carcinogen preceding it, if its action is to be revealed (7).

The rat and the guinea pig are known to differ in their response to the carcinogenic hydrocarbons. In the previous analysis of the mechanism of carcinogenesis in mice (4) it was seen that the latent period was a function of the promoting action, whereas the total number of tumors induced was an expression of the initiating action only. Therefore, it might be concluded that, with an effective carcinogen, the latent period in relatively unresponsive species might be altered by effective promotion. Croton oil, however, appears to have no effect in either the rat or the guinea pig, although neither of the experiments could be described as final, in view of the poor health of the animals.

The results suggest that epidermal hyperplasia and tumor-promoting activity are not related in any simple fashion. Several of the substances investigated in the mouse, other than croton oil, induced marked hyperplasia, and yet were ineffective in promoting tumors following a single application of the carcinogen. It must be mentioned, however, that none of these substances seemed to induce as great a hyperplasia, and with such speed, as that encountered with croton oil, although this aspect requires further and more detailed histologic investigation. Nevertheless, this investigation raises the possibility of a specific metabolic role for croton oil in the mouse, rather than any simple explanation, as hitherto envisaged but not discussed. The only confusing factor is the undoubted effectiveness of wound healing as a promoting agent in the rabbit under these same conditions.

## SUMMARY

1. Turpentine, acridine, fluorene, phenanthrene, castor oil, ricinoleic acid, glyceryl monoricinoleate, oleic acid, and silver nitrate have been tested for tumor-promoting activity following a single application of 9,10-dimethyl-1,2-benzanthracene in the mouse. They were ineffective as promoters of tumors.

2. The effects of wound healing, turpentine and croton oil applications, following a single application of 9,10-dimethyl-1,2-benzanthracene, have been tested for tumor-promoting activity on the rabbit. Wound healing alone has been found to be an effective stimulus.

3. The effects of repeated applications of croton oil, following a minimal number of applications of 9,10-dimethyl-1,2-benzanthracene have been investigated in the guinea pig. No tumors were induced.

4. The effects of croton oil, following three applications of 9,10-dimethyl-1,2-benzanthracene in the rat, have been investigated. No tumors were induced.

5. The specific effect of croton oil on the mouse and of wound healing on the rabbit, as promoters of tumor formation are discussed.

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# Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes

## II. 3′-Methyl-, 2′-Methyl-, and 2-Methyl-4-dimethylaminoazobenzene, 3-Methyl-4-monomethylaminoazobenzene, and 4′-Fluoro-4-dimethylaminoazobenzene

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Previous studies (13, 15) have shown that the compositions of the nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions from liver tumors induced by 4-dimethylaminoazobenzene differ considerably from those of normal livers. The tumors contain more desoxypentosenucleic acid and protein in the nuclear fraction; less pentosenucleic acid, riboflavin, and protein in the large granule fraction; less pentosenucleic acid and protein in the small granule fraction; and much more pentosenucleic acid in the supernatant fluid than do normal livers. Most of these changes are also observed, although to a lesser degree, in the livers of rats fed 4-dimethylaminoazobenzene for only 1 month (13) and to a marked degree in the livers of rats fed the more active 3′-methyl derivative for the same length of time (14). On the other hand, ingestion of the noncarcinogenic dye 4-aminoazobenzene or the weakly active carcinogen 4′-methyl-4-dimethylaminoazobenzene causes little change in the composition of the liver (14).

The data presented here extend these studies to the livers of rats fed the remaining C-monomethyl dyes, 2′-methyl- and 2-methyl-4-dimethylaminoazobenzene and 3-methyl-4-monomethylaminoazobenzene;[1] the relative carcinogenicities of these compounds are 2–3, 0, and less than 1, respectively, when 4-dimethylaminoazobenzene is assigned an activity of 6 (11). 4′-Fluoro-4-dimethylaminoazobenzene (12, activity = 10) was also studied and 3′-methyl-4-dimethylaminoazobenzene (activity = 10–12) was included as a control. In addition, the effect of increasing the casein content of the diet from 12 to 24 per cent on the composition of the livers from rats fed the basal diet with or without 3′-methyl-4-dimethylaminoazobenzene was investigated.

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[1] 3-Methyl-4-monomethylaminoazobenzene has approximately the same carcinogenic activity as the corresponding N-dimethyl compound and was much easier to prepare (11).

### METHODS

These experiments were conducted in two separate but identical series. For each series six groups of male rats[2] weighing 195–220 gm. were fed *ad libitum* a semisynthetic diet (8, diet 3) containing 12 per cent of casein and 1.2 mg. of riboflavin per kilogram. One group was fed the basal diet (no dye added), while the others were fed diets containing either 0.064 per cent of 3′-methyl-4-dimethylaminoazobenzene or equimolar levels of one of the other dyes (10, 11). In the second series two additional groups were fed a 24 per cent casein diet containing either no dye or 0.064 per cent of 3′-methyl-4-dimethylaminoazobenzene. The additional 12 per cent of casein was added at the expense of glucose, and it was a low vitamin type,[3] so that the riboflavin intake was essentially the same for the groups fed either level of casein. Four weeks after the rats were placed on the experimental diets, the group receiving 3′-methyl-4-dimethylaminoazobenzene in the 12 per cent casein diet was

[2] Obtained from the Holtzman Rat Company, Madison, Wisconsin.

[3] "Vitamin Test Casein," S. M. A. Co., Chagrin Falls, Ohio; this preparation contained about 1 μg riboflavin per gram.

killed, and the rest of the animals were killed at 2-day intervals in the order of decreasing carcinogenicities of the dyes fed.

For each fractionation four rats were killed with ether and their livers perfused with saline *in situ*. The pooled livers were forced through a plastic tissue mincer, homogenized in 0.88 M sucrose, and fractionated as previously described (13–15). Each fraction and the whole homogenate were analyzed for protein, desoxypentosenucleic acid, pentosenucleic acid, and protein-bound aminoazo dye as previously described (13); and the average recoveries of these substances in the fractions (sum of the amounts in the four fractions) were 98, 98, 95, and 105 per cent, respectively, of the amounts found in the whole homogenates. In these series riboflavin was determined fluorometrically by the method of Conner and Straub (3), since in our hands this determination was more reproducible than the microbiological assay used previously. In a direct comparison of the two methods, the microbiological assay values averaged 78 per cent of the fluorometric values on the livers of rats fed either the basal diet or diets containing 4-dimethylaminoazobenzene or one of the C-monomethyl dyes; the recoveries of riboflavin in the fractions averaged 102 per cent of the amounts found in the whole homogenates.

For the nuclear counts an aliquot of the freshly prepared homogenate was diluted with 1–3 volumes of 0.88 M sucrose solution, and the resulting suspension was diluted 1–20 in a blood-diluting pipette with 3 per cent acetic acid containing 0.02 per cent of methyl green. The final suspension contained about 50 nuclei in 0.1 cu. mm. The nuclei were counted in a hemocytometer using the standard procedure for the enumeration of white blood cells (5).

The procedure employed for revealing the large granules or mitochondria in tissue sections was essentially that of Cain (2), except that the excised whole livers were fixed by perfusion with Regaud's (6) fixative. In our hands this fixative was superior to Helly's fixative. Since the sections were not improved by postchroming, they were imbedded in paraffin after 8 hours of fixation and 18 hours of washing in water. They were cut at 2 $\mu$ and mounted with the aid of a small amount of albumin. A modification of Cain's method of staining mitochondria was also devised for use with homogenates and the isolated fractions of the cells. Since the entire procedure was carried out in a test tube without transfers, it was possible to do the fixation, washing, and staining with very little loss of tissue. To change from one reagent to another the particles were sedimented in a centrifuge, the supernatant fluid was discarded, and the particles were resuspended in the next reagent. In this procedure 0.2 cc. of a 12 per cent homogenate, or an equivalent quantity of protein from one of the cell fractions, was fixed in 10 cc. of Helly's fixative (6) for 4 hours at room temperature, treated with saturated potassium dichromate for 2 hours, and washed with water until the excess dichromate was removed. The precipitate was stained with 3 cc. of Altmann's aniline acid fuchsin for 5 minutes at 65° C. in a water bath and washed with water until the washings were almost colorless. The differentiation could be accelerated by the use of dilute sodium carbonate as recommended for sections (2). After the nuclei were counterstained with 2 cc. of 0.5 per cent methyl blue for 2 minutes, the excess stain was removed by washing with water, and the precipitate was dehydrated with alcohol and cleared with xylol. Permanent mounts were made by mixing the cleared suspension of particles in xylol with mounting medium and covering with a cover slip. With this procedure the nuclei were blue, the nucleoli red, and the mitochondria appeared as short red rods. The few intact cells present in the homogenates and nuclear fractions were readily identified with this staining procedure.

Slices of each liver used in these experiments were also fixed in 4 per cent formaldehyde, and sections were stained with hematoxylin and eosin (6).

## RESULTS

*Protein distribution.*—The intracellular distribution of protein in the livers of rats fed the basal diet was similar to that observed previously (13, 14), and increasing the level of dietary casein from 12 to 24 per cent failed to alter significantly the protein content of the fractions (Table 1). The ingestion of the most active carcinogen, 3′-methyl-4-dimethylaminoazobenzene, produced essentially the same changes in intracellular protein distribution as were observed previously (14), whether it was fed in the diet containing 12 or 24 per cent of casein. These changes consisted of an average increase of 92 per cent in the amount of nuclear protein and average decreases of 51 and 32 per cent in the protein contents of the large and small granules, respectively. Ingestion of 4′-fluoro-4-dimethylaminoazobenzene altered the protein distribution less than 3′-methyl-4-dimethylaminoazobenzene, even though it is almost as active a carcinogen (12). There was no significant increase in the amount of nuclear protein, and the decreases in the protein contents of the large and small granules averaged only 12 and 15 per cent, respectively. The relatively weak dye 2′-methyl-4-dimethylaminoazobenzene decreased the protein

content of the large granules by an average of 18 per cent, while the very weak carcinogen 3-methyl-4-monomethylaminoazobenzene failed to alter significantly the protein content of any fraction. The inactive dye 2-methyl-4-dimethylaminoazobenzene caused average increases in the protein content of the nuclear and large granule fractions of 32 and 65 per cent, respectively, and a slight decrease in the amount in the small granule fraction. azobenzene doubled the amount of this nucleic acid in the nuclear fraction, while 4′-fluoro-4-dimethylaminoazobenzene caused an average increase of 24 per cent. In spite of the wide differences in the desoxypentosenucleic acid contents of the nuclear fractions from the livers of rats fed the various dyes, the level of this nucleic acid per gram of nuclear protein was relatively constant except for the lower ratio of desoxypentosenucleic acid to

TABLE 1

DISTRIBUTION OF PROTEIN IN THE LIVER FRACTIONS*

| FRACTION | AZO DYE FED | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | | 3′-Me-DAB† | | 4′-F-DAB | 2′-Me-DAB | 2-Me-DAB | 3-Me-MAB‡ |
| | Per cent of casein in the diet | | | | | | | |
| | 18 | 24 | 18 | 24 | 18 | 18 | 18 | 18 |
| | Milligrams of protein per gram of fresh tissue§ | | | | | | | |
| W. Hom.‖ | 137–129 | 144 | 122–119 | 117 | 127–121 | 118–115 | 150–143 | 121–119 |
| Nuclei | 14–20 | 21 | 31–33 | 40 | 17–19 | 17–16 | 23–21 | 14–14 |
| L. Gran.‖ | 40–35 | 43 | 22–20 | 15 | 34–32 | 30–31 | 63–60 | 38–34 |
| S. Gran.‖ | 19–21 | 21 | 14–14 | 13 | 17–17 | 18–20 | 17–17 | 18–20 |
| Sup. Fl.‖ | 56–51 | 56 | 52–51 | 49 | 55–50 | 46–47 | 49–43 | 49–48 |
| Rec.‖ | 129–127 | 140 | 119–118 | 117 | 123–118 | 111–14 | 152–141 | 119–116 |

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.
† DAB = 4-Dimethylaminoazobenzene.
‡ MAB = 4-Monomethylaminoazobenzene.
§ The figures to the nearest whole number.
‖ These abbreviations refer to the whole homogenate, large granules, small granules, supernatant fluid, and recovery, respectively.

TABLE 2

DISTRIBUTION OF DESOXYPENTOSENUCLEIC ACID IN THE LIVER FRACTIONS*

| FRACTION | AZO DYE FED | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | | 3′-Me-DAB† | | 4′-F DAB | 2′-Me-DAB | 2-Me-DAB | 3-Me-MAB‡ |
| | Per cent casein in the diet | | | | | | | |
| | 18 | 24 | 18 | 24 | 18 | 18 | 18 | 18 |
| | Milligrams of nucleic acid per gram of fresh tissue | | | | | | | |
| W. Hom.‖ | 1.83–1.92 | 1.98 | 3.58–3.98 | 4.58 | 2.30–2.29 | 1.84–1.96 | 1.62–1.70 | 1.53–1.92 |
| Nuclei | 1.81–1.84 | 2.07 | 3.45–3.74 | 4.39 | 2.22–2.29 | 1.85–1.84 | 1.55–1.61 | 1.58–1.81 |
| Rec.‖ | 1.81–1.84 | 2.07 | 3.45–3.74 | 4.39 | 2.22–2.29 | 1.85–1.84 | 1.55–1.61 | 1.58–1.81 |
| | Milligrams of nucleic acid per gram of protein§ | | | | | | | |
| W. Hom. | 13–15 | 14 | 29–32 | 39 | 18–19 | 16–17 | 11–12 | 13–16 |
| Nuclei | 129–92 | 99 | 112–113 | 110 | 130–120 | 109–115 | 67–77 | 113–129 |

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.
† DAB = 4-Dimethylaminoazobenzene.
‡ MAB = 4-Monomethylaminoazobenzene.
§ The figures to the nearest whole number.
‖ These abbreviations refer to whole homogenate and recovery, respectively.

*Desoxypentosenucleic acid distribution.*—This nucleic acid was always found in the nuclear fraction only (Table 2). Ingestion of the inactive dye 2-methyl-4-dimethylaminoazobenzene, the relatively inactive compound 3-methyl-4-monomethylaminoazobenzene, or the moderately active carcinogen 2′-methyl-4-dimethylaminoazobenzene did not alter appreciably the desoxypentosenucleic acid content of the nuclear fraction. On the other hand, consumption of 3′-methyl-4-dimethylaminoprotein in the nuclear fraction of the livers from rats fed 2-methyl-4-dimethylaminoazobenzene.

*Pentosenucleic acid distribution.*—Ingestion of any of these dyes altered the levels of pentosenucleic acid in the liver cell fractions, but in general these changes did not correlate well with the carcinogenic potencies of the dyes fed (Table 3). The large granule fractions from the livers of the rats fed either 3′-methyl-4-dimethylaminoazobenzene or any of the other dyes contained only about 40

and 60 per cent, respectively—as much pentosenucleic acid as the same fraction from the control livers. The pentosenucleic acid content of the small granules was also reduced by approximately 35 per cent when any of these dyes were consumed, although these decreases appeared to be slightly smaller when the 4′-fluoro or 2′-methyl derivatives of 4-dimethylaminoazobenzene were fed. In addition, the livers from the rats fed 3′-methyl-4-dimethylaminoazobenzene contained increased amounts of this nucleic acid in both the nuclear and supernatant fluid fractions. In general, the ratio of pentosenucleic acid to protein in the large and small granules was decreased when the dyes were fed; this was particularly striking for the large granules of the livers from the rats fed 2-methyl-4-dimethylaminoazobenzene.

*Riboflavin distribution.*—The total level and intracellular distribution of hepatic riboflavin were very similar whether the basal diet contained 12 or 24 per cent of casein (Table 4). Similarly, the alterations in riboflavin distribution brought about by the ingestion of 3′-methyl-4-dimethylaminoazobenzene were not influenced by the level of dietary casein. When this dye was fed, the large granule fraction contained only 40 per cent as much riboflavin as the same fraction from the livers of rats fed the basal diets. The large granule fractions from the livers of rats fed the 4′-fluoro or 2′-methyl derivatives of 4-dimethylaminoazobenzene or 3-methyl-4-monomethylaminoazobenzene contained, on an average, only 91, 70, and 70 per cent, respectively, as much riboflavin as the same fraction from the control rats. The large granule fraction from the livers of rats fed 2-methyl-4-dimethylaminoazobenzene contained 29 per cent more riboflavin than if the basal diet had been fed. Ingestion of any one of these aminoazo dyes caused decreases of about 38 and 24 per cent in the riboflavin contents of the small granule and supernatant fluid fractions.

*Protein-bound aminoazo dye distribution.*—The distribution of protein-bound aminoazo dye in the liver fractions was very similar, regardless of the dye fed (Table 5). In each case the supernatant fluid fraction contained the highest concentration of bound dye per gram of protein and over half of the protein-bound dye found in the whole liver. The small granules had the second highest concentration of bound dye, while the nuclear fraction generally had the lowest amount per gram of protein. Other studies have shown that the level of bound dye in the livers of rats fed any of these dyes increases for a time and then stabilizes or decreases, even though the dye is fed continuously. The times at which the maximum levels of dye are found have been correlated fairly well with the carcinogenicities of the dyes fed. These maxima are found after approximately 2, 4, 8, 12, and 12 weeks, respectively, when the 3′-methyl, 4′-fluoro, 2′-methyl, and 2-methyl derivatives of 4-dimethylaminoazobenzene and 3-methyl-4-monomethylaminoazobenzene are fed (9, 12). Thus it is apparent that the relative amounts of bound dye found

TABLE 3

DISTRIBUTION OF PENTOSENUCLEIC ACID IN THE LIVER FRACTIONS*

| FRACTION | AZO DYE FED | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | | 3′-Me-DAB† | | 4′-F-DAB | 2′-Me-DAB | 2-Me-DAB | 3-Me-MAB‡ |
| | Per cent casein in the diet | | | | | | | |
| | 12 | 24 | 12 | 24 | 12 | 12 | 12 | 12 |
| | Milligrams of nucleic acid per gram of fresh tissue | | | | | | | |
| W. Hom.‖ | 5.85–5.88 | 6.02 | 4.46–4.24 | 4.82 | 4.45–4.30 | 3.90–3.94 | 4.09–3.84 | 3.64–4.50 |
| Nuclei | 0.54–0.64 | 0.77 | 1.05–0.88 | 1.36 | 0.47–0.39 | 0.52–0.40 | 0.49–0.44 | 0.41–0.49 |
| L. Gran.‖ | 1.91–1.69 | 1.80 | 0.75–0.68 | 0.50 | 1.21–1.12 | 1.02–0.97 | 1.09–1.10 | 1.15–1.35 |
| S. Gran.‖ | 1.80–1.99 | 1.92 | 1.10–0.92 | 1.11 | 1.36–1.44 | 1.20–1.42 | 1.12–1.09 | 1.04–1.33 |
| Sup. Fl.‖ | 1.24–1.20 | 1.27 | 1.47–1.34 | 1.53 | 1.22–1.15 | 1.12–0.91 | 1.05–0.81 | 0.86–1.04 |
| Rec.‖ | 5.49–5.52 | 5.74 | 4.37–3.82 | 4.50 | 4.26–4.10 | 3.86–3.70 | 3.75–3.44 | 3.46–4.21 |
| | Milligrams of nucleic acid per gram of protein§ | | | | | | | |
| W. Hom. | 43–45 | 42 | 37–36 | 41 | 35–36 | 33–34 | 27–27 | 30–38 |
| Nuclei | 39–33 | 37 | 34–27 | 34 | 28–21 | 31–25 | 21–21 | 29–35 |
| L. Gran. | 48–48 | 42 | 34–34 | 33 | 36–35 | 34–31 | 17–18 | 30–40 |
| S. Gran. | 95–95 | 94 | 77–66 | 85 | 80–85 | 67–71 | 66–64 | 58–67 |
| Sup. Fl. | 22–23 | 23 | 28–26 | 31 | 22–23 | 24–19 | 21–19 | 18–21 |

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† DAB = 4-Dimethylaminoazobenzene.

‡ MAB = 4-Monomethylaminoazobenzine.

§ The figures to the nearest whole number

‖ These abbreviations refer to the whole homogenate, large granules, small granules, supernatant fluid, and recovery, respectively.

on analysis of the livers of rats fed a series of dyes will depend on the time at which they are killed as well as on the differences in the maximum levels of bound dye which can be formed.

*Enumeration of nuclei.*—The nuclear counts made on the liver homogenates from the second series of fractionations are presented in Table 6. The figure of 137 × $10^6$ nuclei per gram of liver from the rats fed the basal diet is in good agreement with the data (118–61 × $10^6$ nuclei per gram) of Brues, Drury, and Brues (1), who used other methods of counting. When the strongly carcinogenic dyes 3′-methyl- and 4′-fluoro-4-dimethylaminoazobenzene were ingested, the number of nuclei per gram of liver rose to 336 and 229 × $10^6$, respectively. However, since the level of desoxypentosenucleic acid increased proportionately, the calculated amount of desoxypentosenu-

TABLE 4

DISTRIBUTION OF RIBOFLAVIN IN THE LIVER FRACTIONS*

| FRACTION | AZO DYE FED | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | | 3′-Me-DAB† | | 4′-F-DAB | 2′-Me-DAB | 2-Me-DAB | 3-Me-MAB‡ |
| | Per cent casein in the diet | | | | | | | |
| | 12 | 24 | 12 | 24 | 12 | 12 | 12 | 12 |
| | Micrograms of riboflavin per gram of fresh tissue | | | | | | | |
| W. Hom.‖ | 14.3–11.8 | 12.7 | 8.8–7.6 | 7.7 | 11.8–10.1 | 9.2–9.9 | 18.2–10.9 | 9.4–8.2 |
| Nuclei | 1.5– 1.8 | 1.6 | 1.7–1.8 | 2.5 | 1.3–1.2 | 1.2–1.0 | 2.4– 1.4 | 0.8–0.7 |
| L. Gran.‖ | 7.7– 5.5 | 6.3 | 3.0–2.5 | 2.5 | 6.6– 5.3 | 4.2–4.7 | 10.9– 6.4 | 4.7–4.4 |
| S. Gran.‖ | 2.4– 1.8 | 1.6 | 1.2–0.9 | 1.1 | 1.4– 1.2 | 1.6–1.4 | 1.6– 1.2 | 1.4–1.5 |
| Sup. Fl.‖ | 3.5– 3.1 | 3.2 | 2.9–2.0 | 2.2 | 2.9– 2.5 | 2.4–2.6 | 2.8– 2.2 | 2.3–2.1 |
| Rec.‖ | 15.1–12.2 | 12.7 | 8.8–7.2 | 8.3 | 12.2–10.2 | 9.4–9.7 | 17.7–11.2 | 9.2–8.7 |
| | Micrograms of riboflavin per gram of protein§ | | | | | | | |
| W. Hom. | 104–91 | 88 | 72–64 | 66 | 93–83 | 78–86 | 121–76 | 78–69 |
| Nuclei | 107–90 | 76 | 55–55 | 63 | 75–63 | 70–63 | 104–67 | 57–50 |
| L. Gran. | 193–157 | 146 | 136–125 | 167 | 192–166 | 140–152 | 173–107 | 124–129 |
| S. Gran. | 126–86 | 76 | 84–64 | 85 | 82–71 | 87–70 | 94–71 | 78–75 |
| Sup. Fl. | 62–61 | 57 | 56–39 | 45 | 53–50 | 52–55 | 57–51 | 47–44 |

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† DAB = 4-Dimethylaminoazobenzene.

‡ MAB = 4-Monomethylaminoazobenzene.

§ The figures to the nearest whole number.

‖ These abbreviations refer to whole homogenate, large granules, small granules, supernatant fluid, and recovery, respectively.

TABLE 5

DISTRIBUTION OF PROTEIN-BOUND AMINOAZO DYE IN THE LIVER FRACTIONS*

| FRACTION | AZO DYE FED | | | | | |
|---|---|---|---|---|---|---|
| | 3′-Me-DAB† | | 4′-F-DAB | 2′-Me-DAB | 2-Me-DAB | 3-Me-MAB‡ |
| | Per cent casein in the diet | | | | | |
| | 12 | 24 | 12 | 12 | 12 | 12 |
| | Micromoles × $10^2$ of dye§ per gram of fresh tissue | | | | | |
| Whole homogenate | 3.84–3.59 | 2.95 | 2.82–2.21 | 2.88–1.69 | 5.32–3.72 | 4.33–4.65 |
| Nuclei | 0.49–0.35 | 0.51 | 0.21–0.20 | 0.23–0.08 | 0.25–0.18 | 0.17–0.18 |
| Large granules | 0.47–0.31 | 0.31 | 0.57–0.44 | 0.45–0.40 | 1.34–1.20 | 0.62–0.69 |
| Small granules | 0.69–0.52 | 0.45 | 0.55–0.39 | 0.51–0.39 | 0.59–0.64 | 0.80–0.83 |
| Supernatant fluid | 2.66–2.34 | 1.77 | 1.68–1.37 | 1.52–1.17 | 2.94–1.96 | 2.89–3.03 |
| Recovery | 4.31–3.52 | 3.04 | 3.01–2.40 | 2.71–2.04 | 5.12–3.98 | 4.48–4.73 |
| | Micromoles × 10 of dye per gram of protein | | | | | |
| Whole homogenate | 3.14–3.02 | 2.52 | 2.22–1.83 | 2.44–1.47 | 3.54–2.60 | 3.58–3.91 |
| Nuclei | 1.58–1.07 | 1.28 | 1.23–1.05 | 1.36–0.52 | 1.10–0.88 | 1.23–1.31 |
| Large granules | 2.14–1.54 | 2.04 | 1.68–1.36 | 1.50–1.28 | 2.12–2.00 | 1.64–2.04 |
| Small granules | 4.93–3.71 | 3.49 | 3.24–2.32 | 2.82–1.96 | 3.45–3.76 | 4.46–4.13 |
| Supernatant fluid | 5.11–4.59 | 3.60 | 3.06–2.73 | 3.31–2.48 | 6.01–4.56 | 5.90–6.32 |

* The first and second numbers in each column refer to the first and second series of experiments, respectively, as mentioned in the text.

† DAB = 4-Dimethylaminoazobenzene.

‡ MAB = 4-Monomethylaminoazobenzene.

§ The nonspecific absorption in the bound dye extracts was determined by carrying each of the protein samples from the liver fractions of the rats fed the basal diets through the bound dye determination. The absorption of these extracts at 520 mμ gave the following corrections to be applied as log $I_0/I$ × $10^3$ per 100 mg. of protein: whole homogenate 57, nuclei 68, large granules 42, small granules 62, and supernatant fluid 35. The corrected values for log $I_0/I$ were converted to micromoles of dye as described previously (9, 12).

cleic acid per nucleus remained fairly constant. This suggests that it was the number of nuclei rather than their nucleic acid content which was altered by the ingestion of these carcinogens. When the amounts of pentosenucleic acid, protein, and riboflavin per nucleus were calculated, the values were much less constant than for the desoxypentosenucleic acid content. This was not unexpected, since desoxypentosenucleic acid was found only in the nuclear fraction, while the other components were also present in the cytoplasmic fractions which contaminate the nuclear fraction in varying amounts. Furthermore, the estimation of relatively low levels of pentosenucleic acid in the presence of such large quantities of desoxypentosenucleic acid is subject to large errors (16).

*Morphological changes in the livers.*—Since the dyes were fed for 4 weeks, only incipient gross cells could account for some, but not all (see below), of the changes in intracellular composition of these livers. In particular, the number of nuclei in a unit volume of this tissue certainly exceeds that of normal liver tissue. The proliferation of these cells was also apparently responsible for the gross changes that resembled cirrhosis. There were no appreciable changes in microscopic architecture in the livers of the rats fed the other dyes.

The large changes in the levels of protein in the large granules that occurred when 3′-methyl- and 2-methyl-4-dimethylaminoazobenzene were fed prompted us to stain sections of similar livers for large granules or mitochondria and to compare them with normal livers. Following ingestion of 2-methyl-4-dimethylaminoazobenzene, the parenchymal liver cells contained increased numbers of large granules, while the parenchymal liver cells

TABLE 6

THE EFFECTS OF CERTAIN AMINOAZO DYES ON THE NUMBER OF NUCLEI AND THE CALCULATED LEVEL OF DESOXYPENTOSENUCLEIC ACID PER NUCLEUS IN THE LIVER*

| | Azo dye fed | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | | 3′-Me-DAB† | | 4′-F-DAB | 2′-Me-DAB | 2-Me-DAB | 3-Me-MAB‡ |
| | Per cent casein in the diet | | | | | | | |
| | 12 | 24 | 12 | 24 | 12 | 12 | 12 | 12 |
| Number of nuclei per gram of fresh tissue $\times 10^{-6}$ | 137 | 165 | 336 | 435 | 229 | 160 | 126 | 150 |
| Calculated amount of desoxypentosenucleic acid per nucleus, micrograms, $\times 10^{6}$ | 14.0 | 12.0 | 11.4 | 10.5 | 10.0 | 12.2 | 13.5 | 12.8 |

* These data were obtained from the second series of animals.
† DAB = 4-Dimethylaminoazobenzene.
‡ MAB = 4-Monomethylaminoazobenzene.

nodular cirrhosis was noticed in the livers from rats fed the most carcinogenic dyes, while no gross damage was observed when the other dyes were fed. However, some of the dyes caused considerable changes in the body and liver weights. 3′-Methyl- and 4′-fluoro-4-dimethylaminoazobenzene caused weight losses of 35–41 gm. per rat, and the livers from these rats were correspondingly smaller. On the other hand, 2-methyl-4-dimethylaminoazobenzene caused even larger weight losses of 40–45 gm. per rat, but the livers of these rats weighed as much as those from the group fed the basal diet which permitted weight gains of 52–65 gm. per rat.

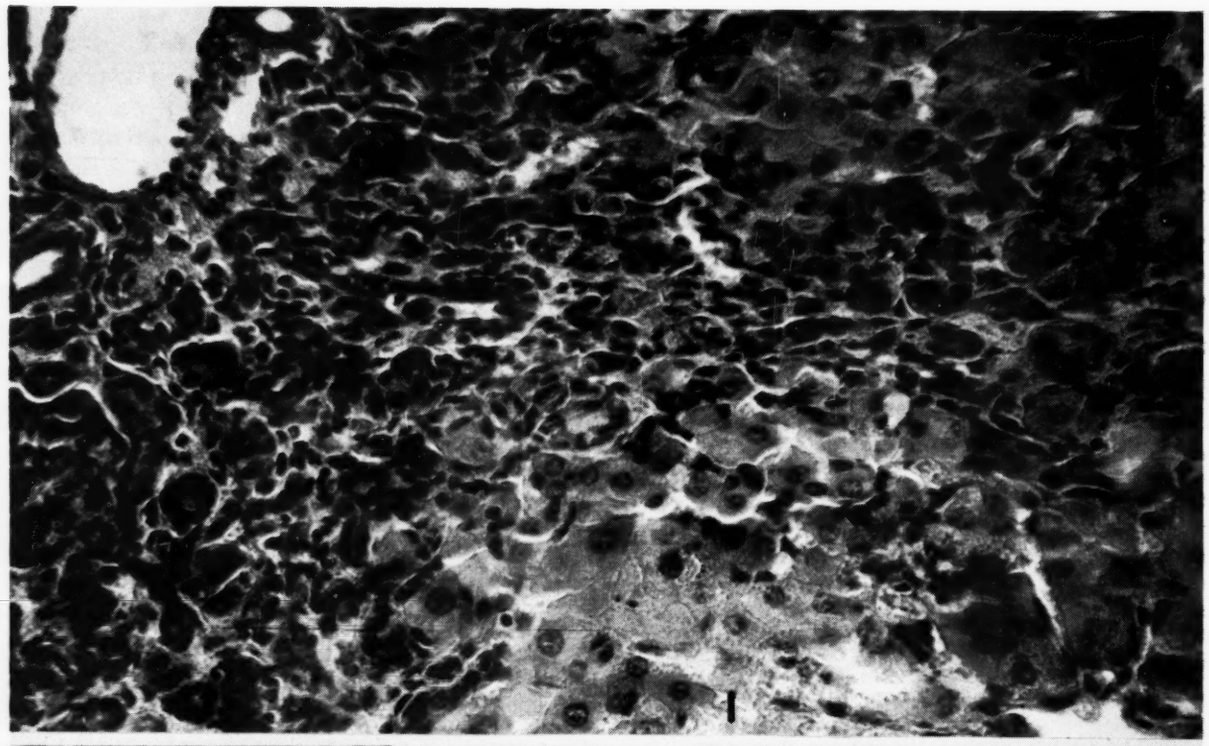
The most striking change observed by microscopic examination of the sections stained with hematoxylin and eosin was a pronounced proliferation of bile duct-like cells in the livers of rats fed 3′-methyl-4-dimethylaminoazobenzene.[4] Figure 1 shows an area in which this change exceeded the average for these livers, but there were a few areas in which even greater changes had occurred. Apparently the proliferation of these small, basophilic from the rats fed the 3′-methyl derivative contained fewer large granules than normal cells (Figs. 2–4).[5] Thus, at least in the case of the large granule fraction, the histological changes observed following ingestion of the dyes appear to be related to the chemical alterations in the parenchymal liver cells.

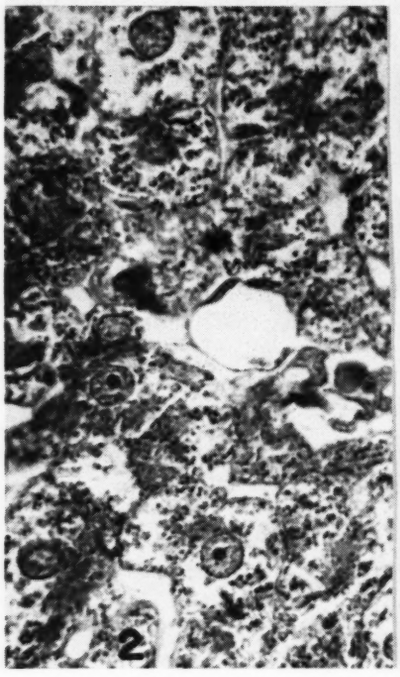
## DISCUSSION

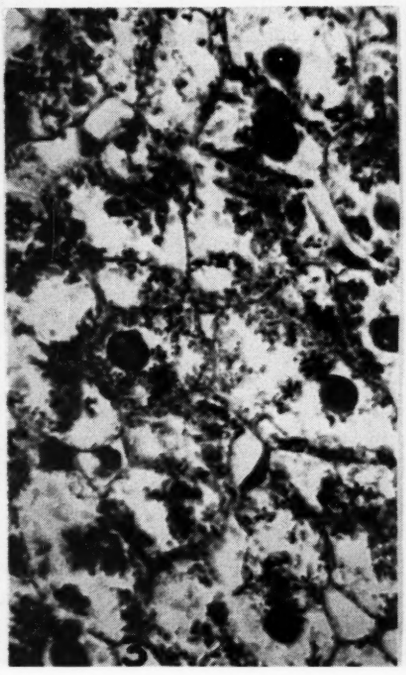
Since ingestion of the azo dyes resulted in certain changes in liver composition (13, 14, and this paper) which can be roughly correlated with the carcinogenic activities of the dyes fed, these changes for the livers of rats fed the C-monomethyl derivatives of 4-dimethylaminoazobenzene and the figures obtained for liver tumors induced by 4-dimethylaminoazobenzene are summarized in

[4] While proliferation of bile duct-like cells is usually found in the livers of rats fed any of the carcinogenic aminoazo dyes until liver tumors develop, the great extent to which these cells proliferate after 3′-methyl-4-dimethylaminoazobenzene has been fed for only 1 month was kindly called to our attention by Dr. Alex B. Novikoff.

[5] We wish to thank Dr. P. L. Gausewitz for aid in the preparation of these photographs.







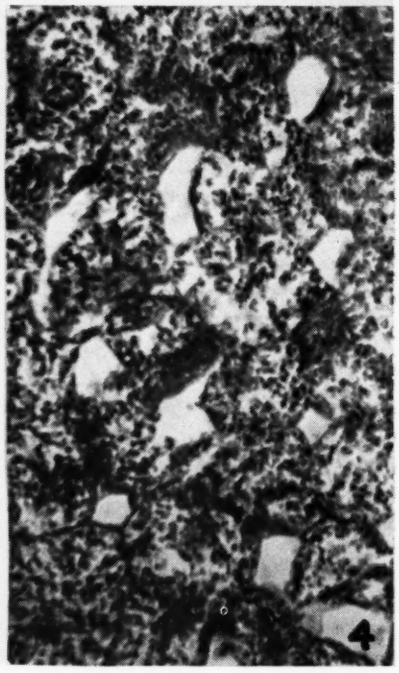


FIG. 1.—Liver from rat fed 0.064 per cent 3′-methyl-4-dimethylaminoazobenzene in the 24 per cent casein diet for 4 weeks, showing replacement of the parenchyma by small, basophilic cells resembling those of the bile ducts. Central vein is shown at lower right. Hematoxylin and eosin stain. × 250.

FIGS. 2–4.—The livers were fixed by perfusion with Regaud's fixative, cut at 2 μ, and stained for mitochondria by the method of Cain.

FIG. 2.—Representative area from liver of rat fed the basal diet. There is some variation in the number of mitochondria in the cells. The nucleoli are unusually prominent in this area. × 700.

FIG. 3.—Representative group of parenchymal liver cells from rat fed 0.064 per cent 3′-methyl-4-dimethylaminoazobenzenes in 12 per cent casein diet for 4 weeks. Most of the cells contain reduced numbers of mitochondria. × 700.

FIG. 4.—Representative area from liver of rat fed 0.064 per cent 2-methyl-4-dimethylaminaozobenzene for 4 weeks. Most of the cells contain more mitochondria than the cells of normal liver. × 700.

Figures 5–8. In each figure the bars representing the levels of the constituent involved and of protein are adjusted to the same length for the livers of rats fed the basal diet; thus it is readily apparent whether the levels of the constituent and protein varied together or independently when a given dye was fed. The carcinogenic potencies of the various dyes are indicated by the numbers above each pair of bars. Each pair of bars represents the average of two to four fractionations; three to four livers or the tumors from six to nine rats were pooled for each fractionation.

Generally, the noncarcinogenic and weakly carcinogenic dyes had little effect on the desoxypentosenucleic acid and protein contents of the nuclear fraction (Fig. 5). However, the noncarcinogenic dye 2-methyl-4-dimethylaminoazobenzene gave rise to nucleic acid and protein values that were consistently lower and higher, respectively, than for the same fraction from the livers of rats fed the basal diet. Since the livers from rats fed this dye had the fewest nuclei per gram of tissue (Table 6) and there was about 65 per cent more protein in the large granule fraction than when no dye was fed (Table 1), it is quite possible that the elevated protein and lowered desoxypentosenucleic acid contents of the nuclear fraction may have been the result of a decreased nuclear/cytoplasmic ratio in the whole liver together with considerable large granule contamination of the nuclear fraction. On the other hand, the active carcinogen 4-dimethylaminoazobenzene produced a small but definite increase in the desoxypentosenucleic acid content of the nuclear fraction, while the 3′-methyl derivative, a very active carcinogen, caused a doubling in the amounts of both desoxypentosenucleic acid and protein. These large increases were due to a great extent to an increase in the number of nuclei per gram of tissue (Table 6). Since a marked proliferation of bile duct-like cells was observed in the livers of rats fed 3′-methyl-4-dimethylaminoazobenzene, a considerable alteration in nuclear types occurred here without appreciably affecting the amount of desoxypentosenucleic acid per nucleus. This is consistent with the data of Vendrely and Vendrely (17) that the nuclei of somatic cells from different tissues of a given species contain the same amount of desoxypentosenucleic acid and that this amount is just double that of the haploid cells (spermatozoa) of the same animal. Our determinations would be subject to error if there was any great change in the degree of polyploidy in the liver or bile duct cells; but most of the increase in desoxypentosenucleic acid in a gram of liver appears to be accounted for by the presence of more nuclei per gram of tissue with each nucleus containing normal or nearly normal amounts of desoxypentosenucleic acid. Although neoplastic tissue was not morphologically evident in stained sections of the livers from the rats fed the 3′-methyl dye for 4 weeks, these livers contained only slightly less desoxypentosenucleic acid per gram than the tumors. In contrast, the 4′-fluoro derivative of 4-dimethylaminoazobenzene, which is almost as carcinogenic as the 3′-methyl derivative, failed to produce such large increases in the protein and desoxypentosenucleic acid content of the nuclear fraction (Tables 1 and 2); this compound caused practically no proliferation of the bile duct-like cells.

In general, when the noncarcinogenic or weakly carcinogenic dyes were fed, the riboflavin and protein contents of the large granules were as high or higher than the levels found in the same fraction from the livers of rats fed the basal diet (Fig. 6); 3-methyl-4-monomethylaminoazobenzene was an exception, since it caused an appreciable reduction in the riboflavin content of this fraction. The more carcinogenic of the C-monomethyl series of dyes appreciably reduced the amounts of protein and riboflavin in this fraction, and in the case of 3′-methyl-4-dimethylaminoazobenzene the levels were almost as low as those found in tumor tissue. However, another strong carcinogen, 4′-fluoro-4-dimethylaminoazobenzene, decreased the protein and riboflavin contents of the large granules only slightly, and not in proportion to its carcinogenic activity when compared with the C-monomethyl dyes. All of the aminoazo dyes that were studied caused some reduction in the pentosenucleic acid content of the large granules, but in general the largest reductions occurred when the most car-

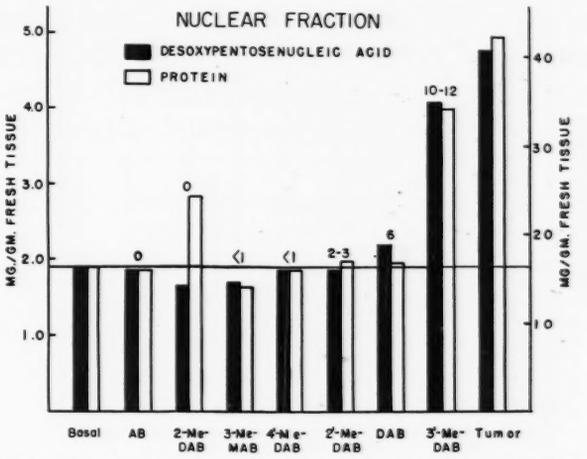



FIG. 5.—Comparison of the levels of desoxypentosenucleic acid and protein in the nuclear fractions from the livers of rats fed various dyes and in tumors induced by 4-dimethylaminoazobenzene. The values in this figure and in figures 6 to 8 are the averages of four fractionations of the livers from rats fed the basal diet and 3′-methyl-4-dimethylaminoazobenzene and of liver tumors, three fractionations of the livers of rats fed 2-methyl-4-dimethylaminoazobenzene, and two fractionations of the livers of rats fed the remaining dyes.

cinogenic dyes were fed (Fig. 7); in the case of the livers from the rats fed 3′-methyl-4-dimethylaminoazobenzene the content was very similar to that of tumor tissue.

It is probable that the changes in the levels of riboflavin and protein in the large granule fraction

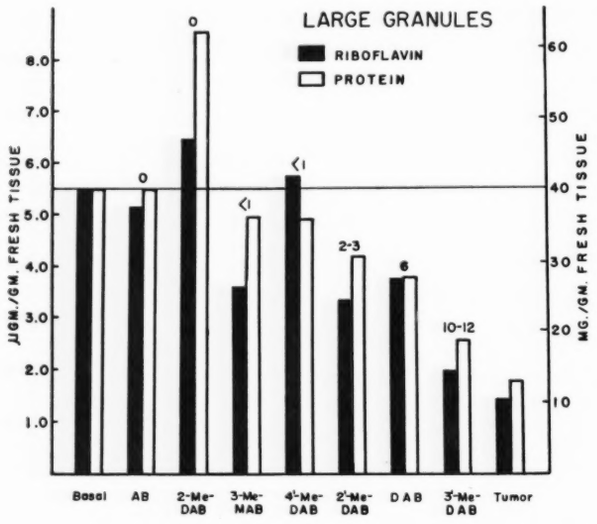



FIG. 6.—Comparison of the levels of riboflavin and protein in the large granule fractions from the livers of rats fed various azo dyes and in tumors induced by 4-dimethylaminoazobenzene. All of the values for riboflavin were obtained by microbiological assay except for the livers from rats fed 2-methyl-4-dimethylaminoazobenzene, 3-methyl-4-monomethylaminoazobenzene, and 2′-methyl-4-dimethylaminoazobenzene; these values were obtained by fluorometric analysis and were recalculated to take into account the differences between the two assays (see Methods).

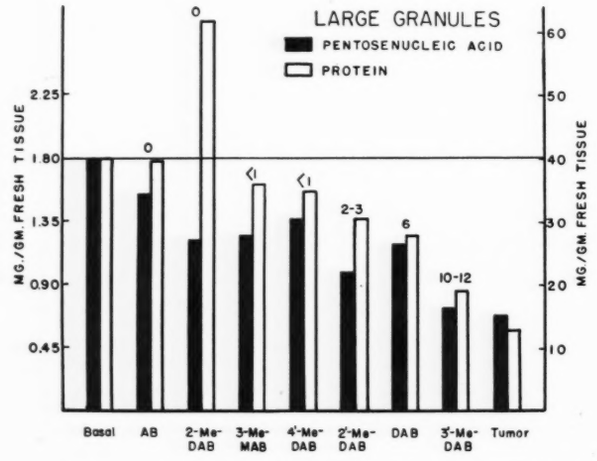



FIG. 7.—Comparison of the levels of pentosenucleic acid and protein in the large granule fractions from the livers of rats fed various azo dyes and in tumors induced by 4-dimethylaminoazobenzene.

were largely the result of changes in the numbers of large granules per cell rather than of gross changes in the qualitative composition of these particles. There were, however, qualitative changes in the large granules from some of the livers, e.g., the great decrease in the ratio of pentosenucleic acid to protein when 2-methyl-4-dimethylaminoazobenzene was fed. This dye is not carcinogenic and is quite toxic (10); in the present experiments it caused a greater average weight loss than any other dye in the series. The effect of this toxic substance on the large granules or mitochondria of the liver is apparently unusual, since Cowdry (4), in a review of the effects of various toxic substances on mitochondria, pointed out that "a diminution in the amount of mitochondria is often encountered, but an increase above normal is comparatively rare."

The protein contents of the supernatant fluids from the livers of rats fed the basal or dye-containing diets or from liver tumors were essentially the same (Fig. 8). Similarly, the pentosenucleic

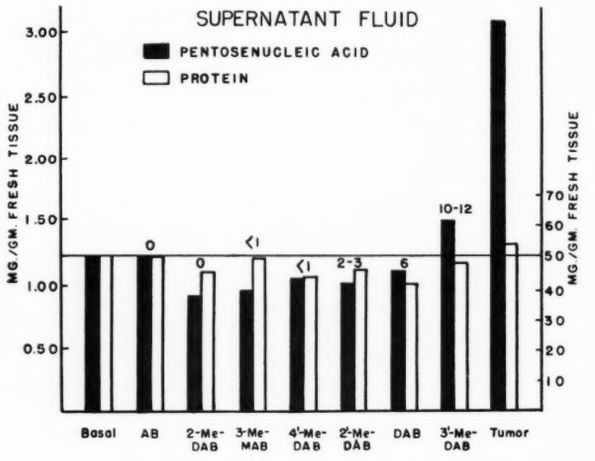



FIG. 8.—Comparison of the levels of pentosenucleic acid and protein in the supernatant fluid fractions from the livers of rats fed various azo dyes and in tumors induced by 4-dimethylaminoazobenzene.

acid content of this fraction was unchanged by the ingestion of any of these dyes except the 3′-methyl derivative. The amount of this nucleic acid in the supernatant fluid from tumor tissue was twice that found in normal liver, with a resultant increase in the ratio of nucleic acid to protein.

Thus it appears that the magnitudes of certain of the changes in intracellular composition induced by 4-dimethylaminoazobenzene and its C-monomethyl derivatives were roughly proportional to the carcinogenic potencies of the dyes. While the alterations induced by the dyes made the compositions of the livers more similar to that of liver tumors, even the most carcinogenic dye in this series, 3′-methyl-4-dimethylaminoazobenzene, failed to cause greater changes from normal liver than were found in the tumor tissue. Further studies will be needed to determine why the changes produced in the liver by ingestion of 4′-fluoro-4-dimethylaminoazobenzene, which is only a little less

carcinogenic than 3′-methyl-4-dimethylaminoazobenzene, are considerably less drastic than the alterations in liver composition induced by the latter dye.

## SUMMARY

1. The livers of rats fed either no azo dye or equimolar levels of 2′-, 3′-, or 2-methyl-4-dimethylaminoazobenzene, 3-methyl-4-monomethylaminoazobenzene, or 4′-fluoro-4-dimethylaminoazobenzene for 4 weeks were perfused, homogenized, and separated by differential centrifugation into nuclear, large granule, small granule, and supernatant fluid fractions. The original homogenate and the fractions were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dye. The results obtained were compared with previous data on the livers of rats fed other aminoazo dyes and on the tumors induced by 4-dimethylaminoazobenzene.

2. From these data it appears that the ingestion of 4-dimethylaminoazobenzene and its C-monomethyl derivatives caused increases in the amounts of protein and desoxypentosenucleic acid in the nuclear fraction; decreases in the amounts of protein, pentosenucleic acid, and riboflavin in the large granule fraction; and increases in the pentosenucleic acid content of the supernatant fluid fraction, which were roughly proportional to the carcinogenicities of the dyes fed. The weak carcinogen 3-methyl-4-monomethylaminoazobenzene was an exception in that it caused a significant reduction in the riboflavin content of the large granules. The changes caused by the strong carcinogen 3′-methyl-4-dimethylaminoazobenzene were not altered by raising the level of dietary casein from 12 to 24 per cent. 4′-Fluoro-4-dimethylaminoazobenzene did not fit into this series, since it failed to cause as great changes as would be expected from its high activity.

3. 2-Methyl-4-dimethylaminoazobenzene differed from the other dyes in that it caused an increase above normal in the number and total mass of the large granules; this fraction contained considerably more protein and riboflavin and less pentosenucleic acid than normal.

4. Ingestion of any of these dyes gave rise to protein-bound aminoazo dye in each fraction of the liver cell; the highest concentration and greatest fraction of the total were always found in the supernatant fluid.

5. Histological studies suggested that the alterations in the composition of the large granule fractions after ingestion of the dyes were due chiefly to changes in the numbers of large granules in the parenchymal liver cells, while proliferation of the bile duct-like cells may have been responsible for the alterations in the composition of the nuclear fraction. The increase in the amount of nuclear desoxypentosenucleic acid which occurred when highly carcinogenic dyes were fed was largely the result of an increase in the number of nuclei per unit weight of tissue.

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# Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes

## III. Effects on Succinoxidase and Oxalacetic Acid Oxidase*

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From various lines of evidence it has been suggested that normal cells may be converted to cancer cells by a deletion of enzymes that are not essential to the life of the cell (3, 4, 7, 16). The testing of this hypothesis has been attempted by studies of enzyme patterns in normal and tumor tissues (7), and it is extended here to a tissue undergoing carcinogenesis. This study arose from the observation that profound changes in the quantities of both nuclear and cytoplasmic constituents of rat liver, in terms of riboflavin, protein, and nucleic acids, were found to occur when certain structurally related aminoazo dyes were fed (11–13). It had been previously shown that the succinoxidase system of liver is localized in the mitochondria (17) and that the decrease in succinoxidase observed in liver tumor, in comparison with normal liver, is paralleled by a decrease in the amount of mitochondria (18). Moreover, Roskelly *et al.* (16) had shown that in livers undergoing carcinogenesis with 4-dimethylaminoazobenzene there was a progressive loss in the response of tissue slices to succinate following an initial increased response. From these earlier experiments it was anticipated that the succinoxidase contents of the livers of rats fed various aminoazo dyes might show variations comparable to the changes in the amounts of mitochondria found in these livers (13).

In conjunction with the experiments reported in the preceding paper (13), certain enzyme systems were determined in the same tissues. The results obtained with two of the dyes are of interest in connection with the deletion hypothesis. The potent carcinogen 3′-methyl-4-dimethylaminoazobenzene produced decreases in the amounts of mitochondria or large granules (13) and succinoxidase that were so great that the liver resembled liver tumor in this respect; in contrast, 2-methyl-4-dimethylaminoazobenzene, which is noncarcinogenic, actually produced *increases* in the amounts of these constituents. The present report will cover the results obtained with these and related compounds in terms of the succinoxidase system and a closely related system that has been referred to as the oxalacetic acid oxidase system (9).

### METHODS

The details as to the basal diet, dyes, and animals are given in the preceding paper (13). All of the rats were killed by ether anesthesia; accessory studies showed that this did not affect the enzyme assays. One experiment was designed to determine the rates at which the activities of the succinoxidase and oxalacetic acid oxidase systems in the liver changed when the diet was altered. For this purpose 54 rats were fed the stock grain diet for 7 days after arrival in the laboratory; at this time 33 were shifted to the basal diet and 15 to the basal diet containing 3′-methyl-4-dimethylaminoazobenzene; at various times three rats on one of the experimental diets and one rat from the grain diet were killed for analysis. After 19 days, 18 of the 24 rats remaining on the basal diet were shifted to the diet containing 3′-methyl-4-dimethylaminoazobenzene; at intervals, the livers from three rats fed the dye and one rat fed the basal diet were assayed for each enzyme.

In a second experiment, which was co-ordinated

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with the fractionation studies (13), the same enzyme assays were made on the livers of rats which had been fed either the basal diet or this diet plus various aminoazo dyes for 4–6 weeks. When an experimental group was killed, the four livers were perfused *in situ* and pooled for the studies involving centrifugal fractionation (13); but the enzyme studies were carried out on individual samples taken from three[1] of the rats just before perfusion. This was necessary since the perfusion seemed to alter the results considerably in the case of the oxalacetate oxidizing system. The liver from a rat fed the grain diet was also assayed with each group of three experimental rats. This experiment was carried out in two parts using two lots of animals (Ser. I and II) that were placed on the experimental diets several months apart; thus the data on each dye are based upon individual assays of the livers from six rats.

In addition, several groups of rats were studied to gain information on other aspects of the problem. These included rats receiving a diet containing a high (10 mg/kg) level of riboflavin, rats that had been shifted from the basal diet plus the 3′-methyl dye to the basal diet alone, and rats that had been placed upon a basal diet in which the protein had been replaced by carbohydrate. These various groups of rats, as well as the stock rats, were not included in the fractionation studies, and hence the data are segregated from other data in the same table.

*Enzyme assays.*—Succinoxidase activity was measured according to the method of Schneider and Potter, (19), using 20 mg. of liver per flask. The oxalacetic acid oxidase system was studied as described by Potter, Pardee, and Lyle (9) except that the substrate consisted of pyruvate and fumarate in final concentrations of 0.005 M and 0.004 M, respectively.[2] Each flask contained 50 mg. of fresh liver. In most cases the livers appeared quite uniform, and the samples, which usually consisted of about 800 mg. taken from the left lobe, were probably representative aliquots. All of the enzyme assays were performed in duplicate.

## RESULTS

*Rate of decrease in enzyme concentration.*—The curves in Figure 1 show the concentrations of succinoxidase in the livers of rats fed 3′-methyl-4-dimethylaminoazobenzene for various periods of time. It may be seen that, when the rats were taken off the stock diet and shifted to either the basal diet alone or the basal diet plus dye, there was a sharp decrease in the succinoxidase concentration. However, the animals on the basal diet reached a steady state at a $Q_{O_2}$ of about 44 in 3–4 weeks, while the animals that had been shifted to the basal diet plus dye showed a further decline to a value of about 27. The animals that had reached a steady state on the basal diet showed a further decline when transferred to the basal diet plus dye and attained a final value of about 31, which is not significantly different from the value of 27 that was attained by the animals shifted directly from the stock diet. All of these values are significantly lower than the value of 60 observed in the rats that were maintained on the stock diet. The figure also

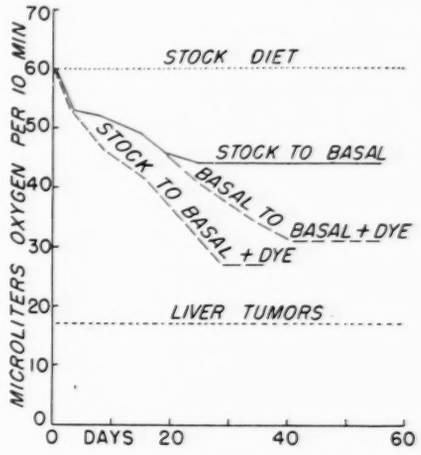


FIG. 1.—Rate of decrease in succinoxidase concentration in livers of rats fed 3′-methyl-4-dimethylaminoazobenzene. The curves are based on the averages of the values in Table 1, plus values for liver tumors obtained earlier (18). Data expressed in terms of oxygen taken up in 10 minutes by 20 mg. fresh weight of liver or tumor.

includes data obtained by Schneider (18) on liver tumors induced by 4-dimethylaminoazobenzene.[3] It may be emphasized that the tumor level represents the value observed in tumors that occurred 4–6 months after the beginning of dye administration and has no relation to the time scale of the figure.

The curves given in Figure 1 are based on the averages of data that are given in greater detail in Table 1. This table also includes all of the assays for the oxalacetic acid oxidase system. In the case of this enzyme system it is doubtful whether any of the groups can be said to differ significantly from the group that was maintained on the stock diet.

[1] Technical limitations prevented the use of all four liver samples.

[2] We wish to thank Dr. G. A. LePage for the adenosine triphosphate used in this assay.

[3] Determinations of succinoxidase on 5 samples of liver tumors produced by the 3′-methyl dye showed an average value of about 12, which is similar to the value of 17 reported in Fig. 1 for tumors produced by 4-dimethylaminoazobenzene (18).

*Changes in enzyme concentration following various dyes.*—The data described above showed that following 3′-methyl-4-dimethylaminoazobenzene, the enzyme changes took place over a period of about 4 weeks and tended to approach a steady state at about this time. Although further work must be done to cover longer periods of time, it seems valid to make comparisons between various dyes, using a standard period of dye administration that was chosen on the basis of results with one dye. The fractionations described in the accompanying paper and the enzyme assays reported in Table 2 were carried out on animals that were fed the dyes for 4–6 weeks. The values obtained from the animals on the basal diet and on the 3′-methyl compound are in close agreement with the data given in Table 1 for comparable animals. This compound again produced a significant decrease in the case of succinoxidase, with a value of about 31 in comparison with the value of 48 for animals fed the basal diet. The results obtained with 2-methyl-4-dimethylaminoazobenzene are of special interest, because this compound produced succinoxidase levels that were much *higher* than the values observed with either the basal diet alone or with the stock diet.[4] In contrast, the other dyes produced either slight decreases or no changes in the succinoxidase levels.

Increasing the casein content of the diet from 12 to 24 per cent produced only a slight increase in the succinoxidase values in the livers of animals receiving either no dye or 3′-methyl-4-dimethylaminoazobenzene. Similarly, little or no increase was found in the succinoxidase values of the livers from rats fed a higher (10 mg. per kg.) level of riboflavin in the basal diets containing either 12 or 24 per cent of casein.

The data on oxalacetic acid oxidase show that almost no change in the amount of this enzyme occurred in the cases in which the succinoxidase values changed most significantly (indicated in boldface type in Table 2). In contrast, the values for the animals that received the extremely weak carcinogen 3-methyl-4-monomethylaminoazobenzene seem significantly lowered.

TABLE 1

RATE OF DECREASE IN ENZYME CONTENT OF LIVER FOLLOWING CHANGE IN DIET

Diet described in accompanying paper (13). The dye used was 3′-methyl-4-dimethylaminoazobenzene.

| | Stock diet | | To basal diet (from stock diet) | | To basal diet + dye (from stock diet) | | To basal diet + dye (after 19 days on basal diet) | |
|---|---|---|---|---|---|---|---|---|
| Days from start of experiment | $Q_{O_2}$* Succinate | $Q_{O_2}$ Pyruvate +fumarate | $Q_{O_2}$ Succinate | $Q_{O_2}$ Pyruvate +fumarate | $Q_{O_2}$ Succinate | $Q_{O_2}$ Pyruvate +fumarate | $Q_{O_2}$ Succinate | $Q_{O_2}$ Pyruvate +fumarate |
| 3 | 65 | 28 | 53(48–56) | 37(35–40) | | | | |
| 3 | 56 | 35 | | | 53(46–57) | 30(27–35) | | |
| 8 | | | 52(47–53) | 27(25–29) | | | | |
| 9 | | | | | 46(43–52) | 24(21–27) | | |
| 13 | 61 | 28 | 49(42–53) | 27(22–32) | | | | |
| 16 | 38 | 37 | | | 41(38–42) | 32(30–34) | | |
| 22 | | | 36 | 27 | | | 37(36–39) | 21(19–22) |
| 28 | | | 52 | 29 | | | 44(40–50) | 20(19–21) |
| 29 | 57 | 36 | | | 27(23–30) | 32(29–34) | | |
| 33 | | | 42 | 24 | | | 34(30–39) | 22(20–23) |
| 36 | 65 | 30 | | | 27(26–29) | 27(23–30) | | |
| 42 | | | 39 | 19 | | | 25(18–31) | 22(12–27) |
| 49 | | | 42 | 25 | | | 29(25–35) | 29(24–32) |
| 56 | | | 52 | 24 | | | 35(26–46) | 32(31–35) |
| Final level reached† | 60(6) | 32(6) | 44(6) | 25(6) | 27(6) | 30(6) | 31(12) | 26(12) |

* The data given are based on oxygen uptake per 20 mg. tissue—10 min. See footnote to Table 2. Single figures represent duplicate determinations on one animal; when figures in parentheses are given they represent the range for three animals and the accompanying number is the average of the three.

† The figure given in each case is the average of the number of animals given in parentheses. When this number is less than the total number of animals in the column, it refers to the animals in the later part of the experiment at a time when rapid change was no longer taking place (cf. Fig. 2).

The data in the lower part of Table 2 show the enzyme contents of livers from animals that were shifted from the diet containing the 3′-methyl dye to the basal diet. The results show that when the dye was withdrawn very little change in the succinoxidase values occurred during a 2-week period. The table also includes data on animals shifted to a protein-free basal diet for 8 days after receiving either the basal diet or the basal diet plus 3′-methyl-4-dimethylaminoazobenzene. The livers of these animals showed lowered succinoxidase values that were comparable to the values found in the livers of animals fed the 3′-methyl dye for 4 weeks. It thus appeared that feeding the dye had an effect similar to that of withdrawing the protein from the diet. The effect of protein levels on the succinoxidase content of the liver has been studied more extensively by Klug *et al.* (2).

[4] Similar and even greater increases in the succinoxidase content of rat livers have been obtained by E. G. Shipley *et al.* (21) in alloxan diabetic rats.

## DISCUSSION

In order to facilitate the discussion of the results in relation to the data of the accompanying paper, the relations between the mitochondrial protein and the oxidative enzymes are indicated in Figure 2 in the form of a bar graph. Examination of the data in the accompanying paper indicated that the bars labeled *M* and *S* to represent mitochondria (mg. protein in *Mitochondria* isolated per gram of fresh liver) and *Succinoxidase* ($Q_{O_2}$ on fresh-weight basis). The scales were adjusted to make these two columns the same height in the case of the control animals on the basal diet without dye. In addition, the graph includes bars representing the $Q_{O_2}$ for

TABLE 2

ENZYME ASSAYS ON WHOLE HOMOGENATES FROM LIVERS OF RATS FED VARIOUS AMINOAZO DYES

The data from the first and second series of the second experiment have been segregated and correspond to the same groups of rats that were used in Table 1 of the accompanying paper (13). In addition, supplementary data from rats on the stock diet and from other groups of rats are included in the lower part of the table.

| Dye | Diet | Series no. | No. of animals | Oxygen uptake $\mu l_{O_2}$/20 mg. tissue/10 min.* With succinate Average | With succinate Range | With pyruvate +fumarate Average | With pyruvate +fumarate Range |
|---|---|---|---|---|---|---|---|
| None | Basal (12 per cent casein) | I | 3 | 54 | 51–56 | 28 | 25–30 |
| | | II | 3 | 41 | 38–43 | 25 | 23–26 |
| None | Basal with 24 per casein | I | 3 | 59 | 52–65 | 31 | 29–32 |
| | | II | 3 | 53 | 49–59 | 29 | 27–31 |
| 3′-Methyl-4-dimethylaminoazobenzene | Basal | I | 3 | 33 | 30–38 | 29 | 26–32 |
| | | II | 3 | 28 | 27–30 | 29 | 27–30 |
| 3′-Methyl-4-dimethylaminoazobenzene | Basal with 24 per cent casein | I | 3 | 39 | 27–50 | 31 | 29–32 |
| | | II | 3 | 28 | 24–32 | 24 | 8–38 |
| 4′-Fluoro-4-dimethylaminoazobenzene | Basal | I | 3 | 51 | 49–53 | 24 | 23–25 |
| | | II | 3 | 46 | 44–49 | 26 | 23–28 |
| 2′-Methyl-4-dimethylaminoazobenzene | Basal | I | 3 | 33 | 31–36 | 20 | 14–25 |
| | | II | 3 | 45 | 40–51 | 22 | 18–26 |
| 2-Methyl-4-dimethylaminoazobenzene | Basal | I | 3 | 101 | 85–110 | 25 | 24–25 |
| | | II | 3 | 109 | 102–113 | 26 | 24–27 |
| 3-Methyl-4-monomethylaminoazobenzene | Basal | I | 3 | 40 | 38–43 | 17 | 16–18 |
| | | II | 3 | 36 | 33–38 | 18 | 17–19 |
| None | Basal + high riboflavin | I | 3 | 52 | 49–57 | 37 | 35–38 |
| | | II | 3 | 50 | 45–55 | 39 | 36–41 |
| None | Basal with 24 per cent casein and high riboflavin | II | 3 | 56 | 53–60 | 29 | 23–31 |
| None | Stock | | 11 | 62 | 52–68 | 31 | 27–37 |
| None | Basal | A† | 3 | 42 | 38–47 | 21 | 17–24 |
| 3′-Methyl-4-dimethylaminoazobenzene‡ | Basal | A† | 2 | 30 | 29–32 | 24 | 22–25 |
| Off 3′-Methyl-4-dimethylaminoazobenzene two days‡ | Basal | A† | 3 | 21 | 18–23 | 18 | 10–23 |
| Off 3′-Methyl-4-dimethylaminoazobenzene five days‡ | Basal | A† | 3 | 26 | 22–30 | 18 | 15–22 |
| Off 3′-Methyl-4-dimethylaminoazobenzene eight days‡ | Basal | A† | 3 | 26 | 20–35 | 25 | 23–28 |
| Off 3′-Methyl-4-dimethylaminoazobenzene 14 days‡ | Basal | A† | 2 | 34 | 32–36 | 33 | 30–35 |
| Off 3′-Methyl-4-dimethylaminoazobenzene eight days | Basal while on dye; followed by protein-free basal | A† | 3 | 27 | 26–27 | 18 | 15–21 |
| None | Basal followed by protein-free basal 8 days | A† | 2 | 32 | 29–34 | 13 | 12–14 |

* This figure is identical with the $Q_{O_2}$ ($\mu l_{O_2}$ per mg. dry weight per hour) in normal liver with a moisture content of 70 per cent.

† Samples were from perfused livers.

‡ All animals received basal diet plus dye for four weeks, then were placed on basal diet for the period indicated.

enzyme changes were not correlated with any of the other constituents of the liver cells for which analyses were made. As might be expected, a close correlation was found to exist between the succinoxidase content and the mass of the mitochondria. The figure therefore was constructed with adjacent the *Oxalacetic acid oxidizing enzyme* labeled *O*. The average values for groups of rats fed four of the C-monomethyl derivatives and the 4′-fluoro derivative of 4-dimethylaminoazobenzene are arranged in the order of increasing carcinogenicity along with the basal diet control values and the

values found in liver tumors induced by 4-dimethylaminoazobenzene.[5] The carcinogenic index at the bottom of the chart gives the relative carcinogenicities of the compounds (5, 6).

In the C-monomethyl series of dyes the compounds appear to decrease the amount of mitochondrial protein and the succinoxidase activity in proportion to their carcinogenicity, thereby producing changes in the liver cells that shift their composition in the direction of the tumor cells.

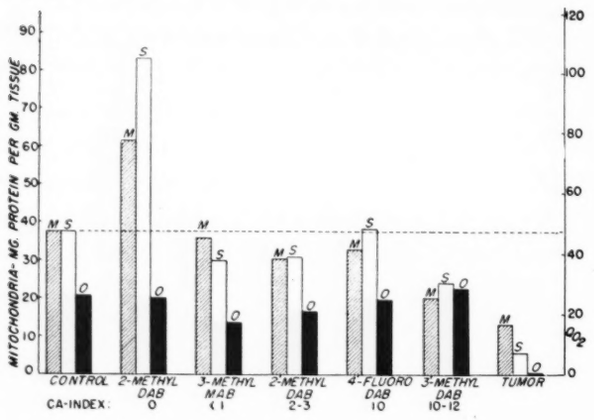


FIG. 2.—Correlations between changes in mitochondria and succinoxidase with the carcinogenicity of aminoazo dyes.

The bars labeled *M*, *S*, and *O* represent, respectively, the weight of *M*itochondrial protein per gram of fresh liver with scale on the left, and the *S*uccinoxidase and the *O*xalacetic Oxidase $Q_{O_2}$ values with scale on the right. The $Q_{O_2}$ values given are the direct figures obtained in terms of microliters of oxygen taken up per 20 mg. of fresh tissue per 10 minutes, which is identical with the conventional $Q_{O_2}$ in normal liver having a dry weight of 30 per cent.

The $Q_{O_2}$ scale was arbitrarily adjusted so as to make the height of the succinoxidase and mitochondrial bars the same in the case of the control group.

The values indicated by the bars represent the averages for the same groups of rats reported in Table 2 and in the accompanying paper. The values for the tumors are taken from independent measurements (18).

The compounds are named as derivatives of 4-dimethylaminoazobenzene (DAB). The "CA-Index" indicates the relative carcinogenicities of the dyes (5, 6).

The 4′-fluoro compound did not fit into the series, since it was strongly carcinogenic but did not lower the succinoxidase values.

The noncarcinogenic dye, 2-methyl-4-dimethylaminoazobenzene, had the remarkable effect of *increasing* the concentration of mitochondria and of succinoxidase[4] above the control levels, while the values for the oxalacetic acid oxidase were no higher than in the controls. Furthermore, the succinoxidase increased to a greater extent than did the mitochondrial protein. These facts suggest either that the composition of the individual mitochondria was altered by the dye or that the distribution of qualitative types in the mitochondrial population was altered. The change in the pentosenucleic acid:protein ratio leads to a similar conclusion (13). It cannot be concluded that the oxalacetic acid oxidase figure is a reflection of mitochondrial changes alone because the rate depends upon other components of the homogenate in addition to the mitochondria (20).

The increase in mitochondria in the case of the 2-methyl-4-dimethylaminoazobenzene suggests that the amount of mitochondria in a given liver cell at any time is the resultant of the independent rates of cell growth or multiplication versus the rates of mitochondrial synthesis and dissolution. The various dyes evidently influence these separate factors quite differently, and the time course of the changes produced by the various dyes may be quite different from the rate of change found for the 3′-methyl compound shown in Figure 1. Consequently, when samples are taken after a fixed time interval, as in Figure 2, it is possible that the opposing factors of mitochondrial synthesis and destruction have varied differently with time when different dyes are given. Further experiments with time as a variable will be required to show whether this may be the explanation for the fact that 4′-fluoro-4-dimethylaminoazobenzene is highly carcinogenic, yet it produced almost no changes in the enzyme values in the time interval studied.

The above considerations are probably relevant to the case of the original dye used in this work, namely, 4-dimethylaminoazobenzene. On various occasions we attempted, in unpublished studies, to obtain data comparable to that of Roskelly *et al.* (16), using, however, the basal diet, as in the present study. The results were inconclusive, and it may be seen in the preceding papers (11–13) that this dye produces much less decisive changes in the mitochondria than does the 3′-methyl dye. It is possible that the dramatic changes observed by Roskelly *et al.* were facilitated by their use of the rice-carrot diet which was probably less favorable for mitochondrial resynthesis than the basal diet used here. Further studies on the point are in progress.

While much further work must be done to prove conclusively that the mitochondrial population can vary independently of cell division, there is at present considerable support for such a view. It is of importance to establish the point because it might provide a relatively simple explanation of the mechanism for the conversion of a normal cell to a cancer cell. Figure 3 illustrates some of the ways in which the mitochondrial population can be varied relative to the cell mass, and other situ-

[5] The data on oxalacetic acid oxidase came from determinations previously reported (8).

ations could be added. The basic mechanism for producing the irreversible change leading to a cancer cell would be a decrease in the *net* rate of production of one type of mitochondria relative to the rate of cell division. At this time the integration of this concept with carcinogenic processes involving mutations or viruses, with biochemical mechanisms of growth control, and with co-carcinogenic processes involving increased rates of cell division cannot be attempted; but two points need to be emphasized. The first is that the development of the above concept was strongly influenced by the studies on *Paramecium aurelia* that have been carried out by Sonneborn (22) and by Preer (10), but it should be pointed out that the concept as it applies to the cancer problem may now be applied directly to the data on liver and is not dependent upon analogies. The second point is that the mechanism of the irreversible change (see Fig. 3) is not necessarily restricted to the mitochondria, although it is implicitly limited to particles or molecules that occur in cells in relatively limited numbers and that originate within the cell.

Finally, the data suggest that in animals that have been fed carcinogenic azo dyes for periods insufficient to produce liver tumors the livers may contain relatively large numbers of altered cells in which the irreversible change to tumor cells has not occurred. For example, in Figure 1 it is clear that in animals that have been on the dye-containing diet for 1 month (which is not long enough to produce liver tumors) the value for succinoxidase cannot be the resultant of the admixture of tumor cells and normal liver cells; such a mixture would have to consist of 75 per cent tumor cells ($Q_{O_2}$ = 17) plus 25 per cent normal liver cells ($Q_{O_2}$ = 60)

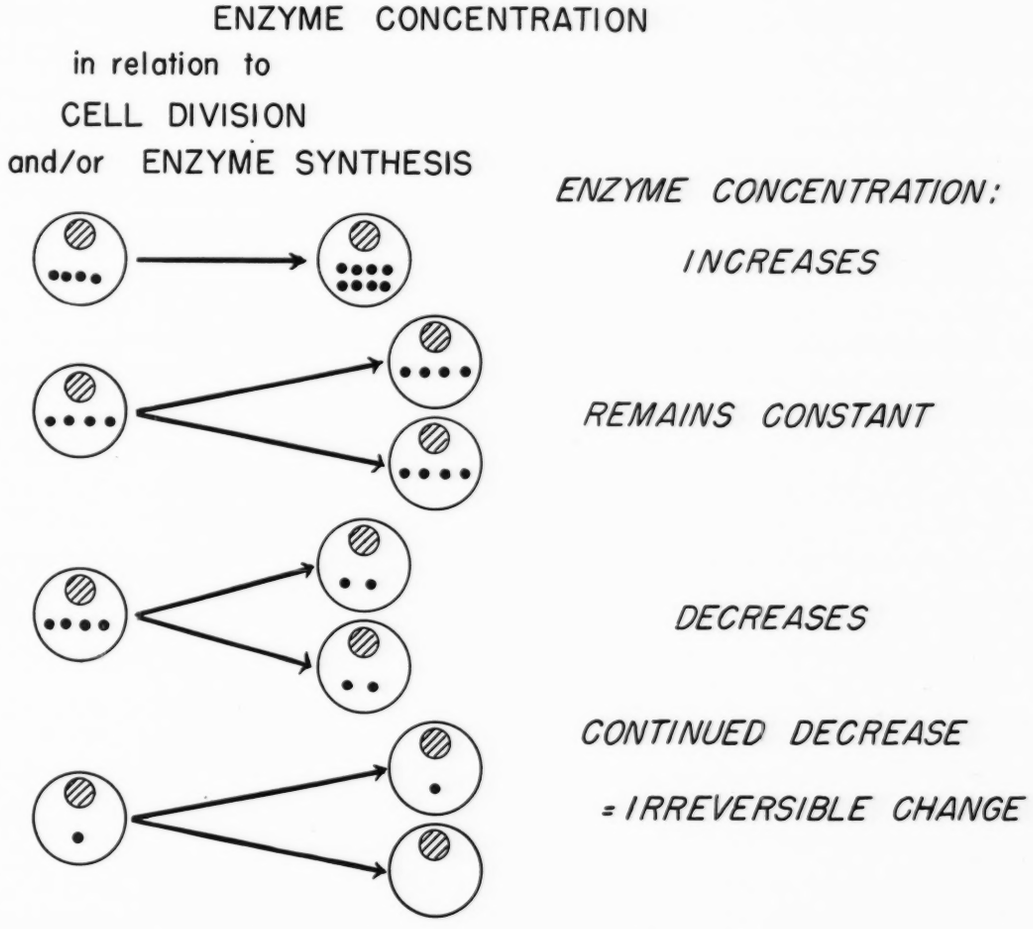


FIG. 3.—Diagrams to illustrate a hypothetical mechanism of carcinogenesis in terms of the deletion of a cytoplasmic particle or an enzyme contained therein. Some possible combinations of rates of particle synthesis and destruction relative to cell division are illustrated as possible explanations for the changes in the amount of mitochondria and enzymes as shown in Fig. 2.

Each large circle represents a cell. The cross-hatched circles represent nuclei, and the small black dots represent *one* type of cytoplasmic particle. The irreversible change is here represented as occurring with the division of a cell containing but one particle, but the work of Preer (10) has shown how the irreversible change could occur in a cell containing more than one particle.

to give the observed $Q_{O_2}$ of 27. Since on the basis of subsequent tumor development it can be concluded that there are few if any viable nests of tumor cells present at this time, it appears that large numbers of liver cells have been considerably changed with respect to their content of mitochondria and succinoxidase. This conclusion is supported by a study of the histological sections (13), which show that the liver cells from animals fed the 3′-methyl dye contain fewer mitochondria than the liver cells of the controls. The sections also show that considerable proliferation of cells resembling bile duct cells had occurred, and this increase in a cell type for which no succinoxidase assays are available adds considerable difficulty to the interpretation of the changes produced by this dye. However, the findings serve to emphasize the need for histological examination of material chosen for biochemical studies, and further work must be done to evaluate their significance.

Finally, it should be noted that the data showing that decreases in mitochondria and succinoxidase can be correlated with carcinogenesis in liver must be evaluated in the light of the excellent studies by Carruthers and Suntzeff (1) and by Roberts and Carruthers (15) on carcinogenesis in skin. It was found that the neoplastic tissue derived from skin had about the same succinoxidase activity as had been reported for liver tumors, but that normal mouse skin had a much lower level. It appears that the tumor cell represents a new enzyme pattern relative to the cells from which it is derived and that the pattern may be approached from opposite directions. Succinoxidase is not considered to be the strategic enzyme system that is deleted, but alterations in its level are possibly a reflection of more subtle changes in the enzyme pattern (cf. 15).

## SUMMARY

1. Assays for the succinoxidase and oxalacetic acid oxidase systems were carried out on the livers of rats that had received a stock grain diet, a basal diet containing 12 per cent casein, or the latter diet containing the 2-methyl, 2′-methyl, 3′-methyl, or 4′-fluoro derivatives of 4-dimethylaminoazobenzene or 3-methyl-4-monomethylaminoazobenzene for periods of 4–6 weeks. In addition, the 3′-methyl compound was fed for shorter periods to determine the time course of the changes in the case of this compound.

2. The results were compared with the analyses for intracellular components reported in the accompanying paper, and the succinoxidase values were found to correlate most clearly with the values for mitochondrial protein, in accordance with the fact that the enzyme is localized in the mitochondria.

3. The succinoxidase values decreased with increasing carcinogenicity within the C-monomethyl series of derivatives but did not decrease significantly with the strongly carcinogenic 4′-fluoro derivative within the period studied.

4. The succinoxidase values increased to more than double the control values in the case of the 2-methyl derivative, but the oxalacetic acid oxidase enzyme system was not increased in the same samples.

5. The results support the view that the synthesis or multiplication of the mitochondria proceeds independently of cell multiplication, and were discussed in relation to the enzyme-deletion hypothesis of cancer formation.

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# Intra-ocular Transplantation of Heterologous Tissues*

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Since Van Dooremaal's (35) report in 1873 concerning the suitability of the anterior chamber as a site for transplantation of tissue, there have been many conflicting reports on the intra-ocular transplantation of heterologous tissues. A few investigators have recently reopened the question and have submitted reports of successful heterologous transplants into the anterior chamber of the eyes of animals with tumor tissue from human beings and other animals. Greene (11) has expressed the opinion that only malignant invasive tumors have the characteristic of heterotransplantability, which serves as a biologic test of malignant disease.

The purposes of our investigation were to determine whether we could successfully transplant tissue from a tumor of a human being into the anterior chamber of the eye of an animal and, if so, to learn (*a*) whether the method could be used as an aid in the diagnosis and prognosis of malignant disease, (*b*) the essential factors for successful transplantation, and (*c*) the biologic factors for growth of the transplant. Our objectives have been partially achieved.

## DEFINITIONS

In our study we defined a homologous transplant as a transplant from one member of a species into another member of the same species.

A heterologous transplant was a transplant from a member of one species into a member of an alien species. The criteria for successful transplantation of tissue should include: (*a*) vascularization of the transplant by the vessels of the host, (*b*) definite growth of the transplant within a reasonable period after transplantation, and (*c*) morphologic and cytologic similarity of the transplant and the tissue originally transplanted. Some observers might also require, and probably justifiably so, eventual metastasis in the host as a necessary criterion of successful transplantation of malignant tumors. Vascularization of a transplant by the host associated with regression of the tumor cannot be considered evidence of successful transplantation.

## RÉSUMÉ OF LITERATURE

*Intra-ocular heterologous transplantation of tumors.*—The history of intra-ocular heterologous transplants may be divided logically into three eras.

The first era, 1884–1912, was a period of agreement among investigators on the nontransplantability of tumors from one species to another. Zahn (37), in 1884, was the first to attempt intra-ocular heterologous transplantation of a tumor. A hyaline enchondroma from a human being was infiltrated with lymphocytes and absorbed after transplantation into the anterior chamber of the eye of the rabbit.

Herzog (16) reported, in 1902, that he had tried for years, without success, to accomplish heterologous intra-ocular transplantation of carcinomas and sarcomas from human beings. Subcutaneous and intraperitoneal heterotransplants were also unsuccessful.

In 1912, Ruben (32) was successful in making homologous transplantation of sarcoma of rats into the anterior chamber and vitreous body, but heterologous transplants into rabbits regressed and failed.

The second era, 1913–1937, was a period of dissension. Hegner (15) and Keysser (20), in 1913, were the first to report successful heterologous transplantation into the anterior chamber. Transplantation of sarcoma of a patient was described as successful in four rats; however, regression occurred later after an unstated period. Transplantation of carcinomatous tissue from human beings failed to take.

Woglom (36), in 1915, was unable to substantiate Hegner's and Keysser's observations. Mouse sarcoma 180 failed to grow in the eyes of any of 83 rats, while 45 takes occurred among 54 control mice.

The third era, 1937 to the present, has been a period of general agreement on the heterotrans-

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plantability of malignant tumors, with views diametrically opposed to those of the first era. Smirnova (34) in 1937 described successful heterotransplantation of carcinomatous tissue from the breast of a human being and of Ehrlich's mouse sarcoma.

Lucké and Schlumberger (23–25), in 1939 and 1940, were the first to report on heterotransplants into the eyes of cold-blooded animals. Carcinomatous tissue from the kidney of the leopard frog was successfully transplanted into members of an alien species. The more closely related the species, the higher the percentage of takes which resulted.

Greene (4), in 1941, reported successful heterologous transplantation of mammary and endometrial carcinomas of the rabbit into the anterior chamber. In the next two years Greene (5, 6) described successful heterotransplants into the anterior chamber of a few tumors from human beings. In 1944, Greene and Lund (8) expressed the opinion that heterotransplantability might be characteristic of cancer. They reported the successful heterologous transplantation of several tumors of human beings into the anterior chambers of guinea pigs.

Greene and Murphy (9), in 1945, reported that heterotransplants of several mouse tumors into the anterior chambers of guinea pigs were successful only after the tumors became invasive in the donor.

Jones, Hale, and Dambach (19), in 1946, recorded successful heterotransplantation into the anterior chamber of an ovarian carcinoma of a human being.

In 1946, Greene (10) reported adoption of heterologous transplantation into the anterior chamber as a routine procedure in his laboratory. He suggested that the guinea pig was superior to the microscope as an aid in the classification of tumors.

Hovenanian, Deming, and Greene reported successful heterologous transplantation into the anterior chamber of two vesical tumors and two urethral carcinomas (2, 17).

Masina (28), in 1947, recorded successful heterotransplants of two renal neoplasms into the anterior chamber. One of his transplants was described as surrounded by a pseudo-capsule. Blake (1) described successful transplantation of a single fibrosarcoma into the anterior chambers of guinea pigs.

Finally, Greene (11) criticized the present concept, which is generally accepted, of diagnosis of neoplasms based on morphologic and cytologic criteria. He suggested that tissues should be assessed according to their behavior as transplants.

Schilling, Snell, and Favata (33) reported successful heterologous transplants of cancerous tissue from human beings into the anterior chamber in 8 of 36 animals, but ultimate regression of these transplants ensued.

Recently Lushbaugh and Steiner (27) reported unsuccessful heterologous transplants into the anterior chamber of several animals of malignant lymphomas from human beings and animals. These transplants underwent rapid necrosis and partial absorption followed by organization of the debris.

*Homologous intra-ocular transplantation of tumors.*—Happe (14), in 1913, achieved successful homologous transplants to the anterior chamber and vitreous body of a round-cell sarcoma of the rabbit which eventuated in metastasis in one animal. In the same year Hegner (15) and Keysser (20) reported metastasis after successful homologous transplantation of mouse carcinoma and sarcoma into the anterior chamber.

In 1928, Gyotoku (12, 13) described successful homologous transplantation into the anterior and posterior chambers of Kato rabbit sarcoma with metastasis to the liver, lungs, and kidneys in some instances.

Greene (3) reported that intra-ocular homologous transplants of mammary tumors of rabbits were successful only after an invasive tumor had developed in the donor. Three years later Greene and Brown (7) described similar results after homotransplantation of a squamous-cell carcinoma of the rabbit.

Lushbaugh and Steiner (27) achieved successful homologous transplants of lymphatic leukemia into the anterior chamber of mice. Local infiltration and distant metastasis occurred in mice of homologous strain, while regression of transplants occurred in most of the mice of heterologous strain.

While not an example of intra-ocular transplantation, one of the most remarkable experiments in homologous transplantation of a tumor was reported by Kurtzahn (21). He transplanted portions of a mammary adenocarcinoma from a human being into the subcutaneous tissue of his own thigh. The transplants became necrotic, infiltrated with lymphocytes and giant cells, and finally were completely destroyed.

*Tissue culture.*—Some appreciation of the behavior of tissue culture is essential for the proper evaluation of the results of this study. Lambert and Hanes (22) demonstrated that various tumors of animals would grow in the plasma of alien species. In 1914, Murphy (29) substantiated the findings of Lambert and Hanes. Lumsden (26) achieved successful tissue culture of several tumors from animals and one carcinoma from the

breast of a woman in the plasma of normal rabbits and sheep. However, tissue cultures were killed by immune serum of an alien species.

## MATERIALS AND METHODS

Tissue from four malignant tumors and one specimen of benign tissue from human beings were transplanted into the anterior chamber of sixteen guinea pigs and within the lens of the eye of five animals. Tissue from 36 malignant tumors, three benign tumors, and one specimen of placental tissue from patients were transplanted solely into the anterior chambers of 170 guinea pigs. All of the specimens were removed surgically from living patients. Carefully selected portions of each specimen were placed in a sterile Petri dish which was kept in the refrigerator until shortly before the transplants. The period of refrigeration was usually one to four hours' duration. Immediate study of frozen sections of tumor tissue contiguous to the portion selected for transplantation was made in order to evaluate the character of the transplant.

TABLE 1

TISSUE FROM MALIGNANT TUMORS AND BENIGN TISSUE OF HUMAN BEINGS TRANSPLANTED INTO THE EYES OF GUINEA PIGS

| Tissue | Type of tissue | Number of transplants | | Successful transplants | |
|---|---|---|---|---|---|
| | | Anterior chamber | Intralenticular | Anterior chamber | Intralenticular |
| 1 | Squamous-cell carcinoma | 3 | 1 | 0 | 1 |
| 2 | Adenocarcinoma | 4 | 1 | 0 | 1 |
| 3 | Fibrosarcoma | 2 | 1 | 0 | 1 |
| 4 | Adeno-acanthoma | 3 | 1 | 1 | 1 |
| 5 | Normal bronchial tissues | 4 | 1 | 0 | 1 |
| Total | | 16 | 5 | 1 | 5 |

## TECHNIC OF TRANSPLANTATION

A sterile technic was employed in all phases of the experiments.

The animals were anesthetized with sodium pentobarbital administered intraperitoneally.

A small piece of tumor is loaded in a trocar. The nictitating membrane is grasped with forceps, which serve to immobilize the globe of the eye. An incision is made in the cornea, near the sclerocorneal junction. The loaded trocar is inserted and advanced to the angle on the opposite side of the anterior chamber. The obturator is depressed, depositing the transplant in the desired location. A cotton applicator stick rolled gently from the incision site across the surface of the cornea toward the site of the transplant secured the transplant in the angle.

Intralenticular transplantation may be accomplished by directing the trocar beneath the capsule of the lens. One eye in each animal was used as the site of the transplant. No further treatment of the eye was necessary, and the animals were kept under ordinary conditions of cage life.

Fixed sections were made of the tissue contiguous to the portions used as transplants as a final control of the character of the tissue transplanted.

The experiments were usually terminated after about 60–70 days of observation. In several instances one member of the series of animals harboring transplants of a given tumor was observed for several months.

Complete necropsies were performed on all animals.

## RESULTS

An appreciation of a few facts concerning the lens of the eye is essential in evaluating the results of this study. The capsule of the lens is a hyaline membrane secreted by a single row of cuboidal cells covering the anterior surface of the lens (30). The capsule is described, in part, in *Gray's Anatomy* (18) as follows: "It is brittle but highly elastic, and when it is ruptured the edges roll up with the outer surface innermost." Small wounds of the capsule may be closed with the formation of a small cataract. Varying amounts of the lenticular fibers may be extruded and may become free masses in the anterior chamber if the wound is large. These masses are gradually dissolved by the aqueous humor and pass out with it through the angle of the chamber. In this manner a large part of the lens, with the exception of the capsule, may be absorbed (31).

*Tissue transplanted both into the anterior chamber and into the lens of the eye.*—The results of this group of transplants are summarized in Table 1. Four intralenticular transplants of malignant tumors and one of benign tissue survived and in some cases grew. The capsule of the lens which surrounded the transplants protected them from vascularization. Consequently, the host could not react to the alien transplants, which were nourished by imbibition of nutrient material through the capsule, just as the lens is normally nourished.

On the other hand, transplants into the anterior chamber were completely absorbed, or showed residual reactions of the host in and about remnants of the stroma of transplant. The tumor cells were destroyed. One exception will be described later.

Thus the fundamental difference observed between transplants into the anterior chamber and intralenticular transplants was vascularization of the former. Avascular transplants within the lens

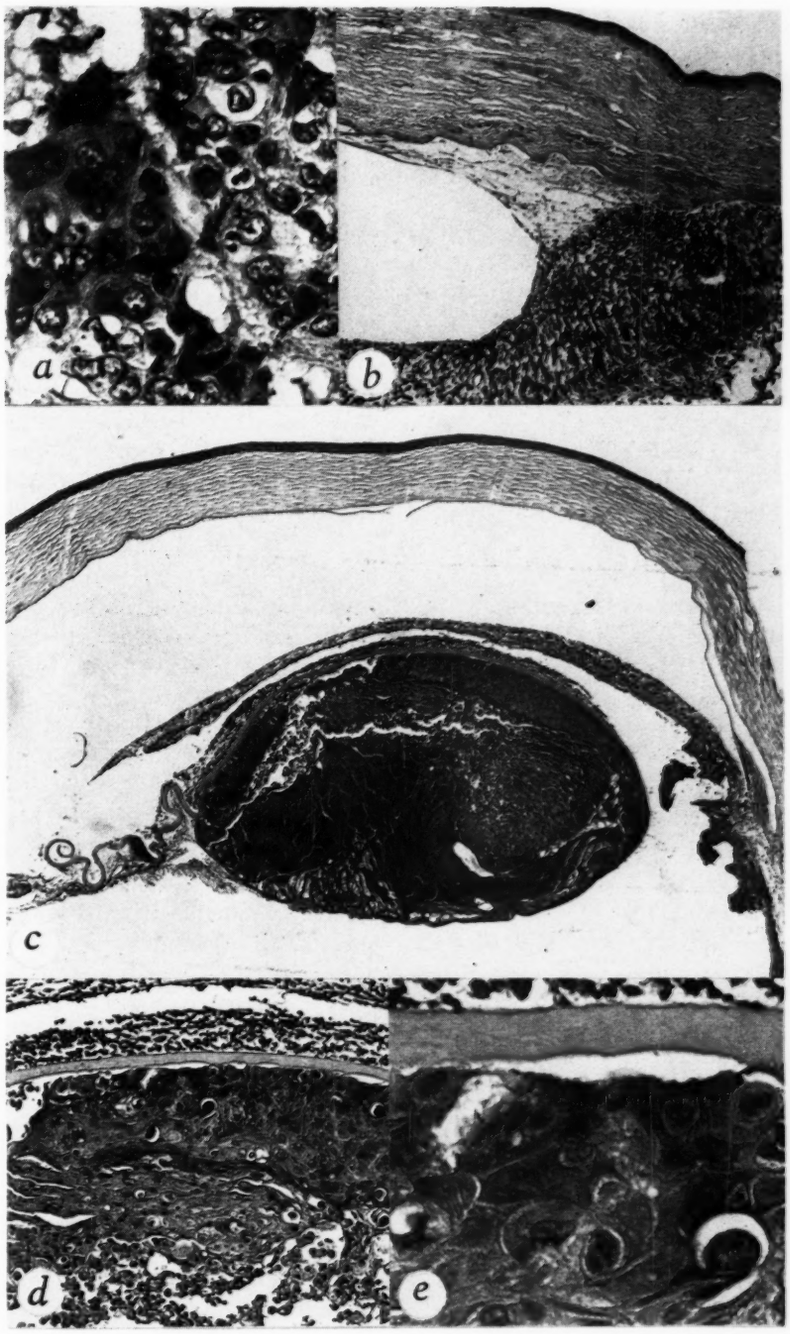


FIG. 1.—Tissue 1. *a*. A squamous-cell epithelioma of the bronchus (hematoxylin and eosin, ×350). *b*. Sixty-four days after transplantation of tissue into the anterior chamber. The cornea appears above, the iris below. The tumor has been destroyed. Note residual lymphocytic infiltration in the angle (hematoxylin and eosin, ×45). *c*. Intralenticular transplant of tissue 1, 63 days after transplantation. The tumor is entirely within the lens. On the left note the characteristic sinuous conformation and rolling of the edge of the ruptured anterior capsule of the lens (hematoxylin and eosin, ×27). *d*. Higher magnification of *c* (hematoxylin and eosin, ×80). *e*. Higher magnification of *d* (hematoxylin and eosin, ×350).

survived and grew as *in vivo* tissue cultures. They are thus comparable somewhat to *in vitro* tissue cultures grown in the serum of alien species.

A squamous-cell carcinoma of the bronchus (Fig. 1, *a*) was transplanted into the anterior chambers of three animals and within the lens of one animal. None of the transplants into the anterior chamber survived. Figure 1, *b*, illustrates the residual lymphocytic infiltration of the remnant of one of the transplants. No tumor cells remained 64 days after transplantation. On the other hand, the intralenticular transplant of the tumor survived 63 days and had grown around the periphery of the lens just beneath the capsule (Fig. 1, *c*, *d*, and *e*). A sinuous conformation and rolling of the edge, characteristic of a fractured lenticular capsule, were present in the left portion of the lens (Fig. 1, *c*). Only a small portion of the lenticular fibers was lost, and the posterior capsule appeared to have closed the defect in the lens.

A papillary adenocarcinoma of the endometrium (Fig. 2, *a*) was transplanted into the anterior chambers of four animals and within the lens of one animal. Three of the transplants in the anterior chamber were completely absorbed, while one underwent calcification and osteogenesis (Fig. 2, *b*). The intralenticular transplant survived 68 days (Fig. 2, *c* and *d*). There was neither vascularization nor cellular infiltration of this transplant. Most of the lenticular fibers were extruded and absorbed by the aqueous humor. The anterior capsule demonstrated the characteristic sinuous outline of a fractured capsule. The fine posterior capsule can hardly be traced, which is usual, in Figure 2, *c*. However, it can definitely be identified in the higher magnification (Fig. 2, *d*).

The animal harboring an intralenticular transplant of fibrosarcoma of the forearm died thirteen days after transplantation. Figure 3 illustrates the surviving transplant completely protected from vascularization and cellular infiltration by the lenticular capsule. Most of the fibers of the lens have been lost to the aqueous humor and absorbed. The serpentine anterior lenticular capsule can be traced, on careful study, throughout its course.

More than 90 per cent of the adeno-acanthoma of the ovary was composed of adenocarcinomatous tissue (Fig. 4, *a*). An intralenticular transplant of this tumor partially survived for 60 days. The transplant was incompletely inclosed by the capsule, and consequently a large part of the tumor was infiltrated with lymphocytes and destroyed. A transplant into the anterior chamber partially survived for 60 days (Fig. 4, *b*). The anterior chamber was filled with an exudate of lymphocytes and some polymorphonuclear leukocytes. Resistant islands of squamous cells survived (Fig. 4, *b* and *c*).

One intralenticular transplant contained tissue from a small-cell carcinoma of the bronchus, as well as normal bronchial elements: cartilage, ciliated columnar epithelium, and mucous glands (Fig. 5, *a*). Sixty-four days after transplantation, an island of viable, benign cartilage and a single space lined with ciliated columnar epithelium (Fig. 5, *b* and *c*) was evident. Almost all of the lenticular fibers were lost. The capsule of the lens had collapsed and inclosed most of the transplant. On the right, where a free edge of the capsule was curled on itself, early cellular infiltration from without was observed. Anteriorly, the damaged lens was adherent to the posterior surface of the cornea. The lenticular capsule could be differentiated readily from Descemet's membrane.

*Results of transplantation solely into the anterior chamber.*—Of transplants from 36 malignant tumors, 3 benign tumors, and 1 placenta in the anterior chamber, none survived. The results of the 170 transplants into the anterior chamber are summarized in Table 2.

Usually transplants in the anterior chambers were completely absorbed in all animals harboring a given tumor. Occasionally, one animal in each series showed some residual host reaction. Cellular infiltration, with either lymphocytes or plasma cells predominating, was observed in remnants of thirteen transplants into anterior chambers. Vascularization and destruction of the tumor tissue had occurred (Fig. 6). In four cases these reactions were incorrectly interpreted as tumors while the animals were living. Fibrosis and hyalinization were observed as residual changes in eleven transplants. None were interpreted as tumors in the living animals. Residual fibrosis and calcification were present in remnants of six transplants, one of which was interpreted as tumor growth before necropsy (Fig. 7). Calcification with osteogenesis occurred in thirteen remnants of transplants, of which six were erroneously interpreted as tumor tissue in living animals.

Residual lymphocytic or plasma-cell infiltration of the iris was observed in 23 animals.

*Early observations after transplantation.*—Early observations after transplantation were limited, since they were not the primary purpose of the study.

The transplant into the anterior chamber from a sarcoma (tumor 10) appeared viable when the animal died, one week after transplantation. Early vascularization was present, but cellular reaction had not occurred around the transplant at this early stage. All the tumor cells in tissue from a semi-

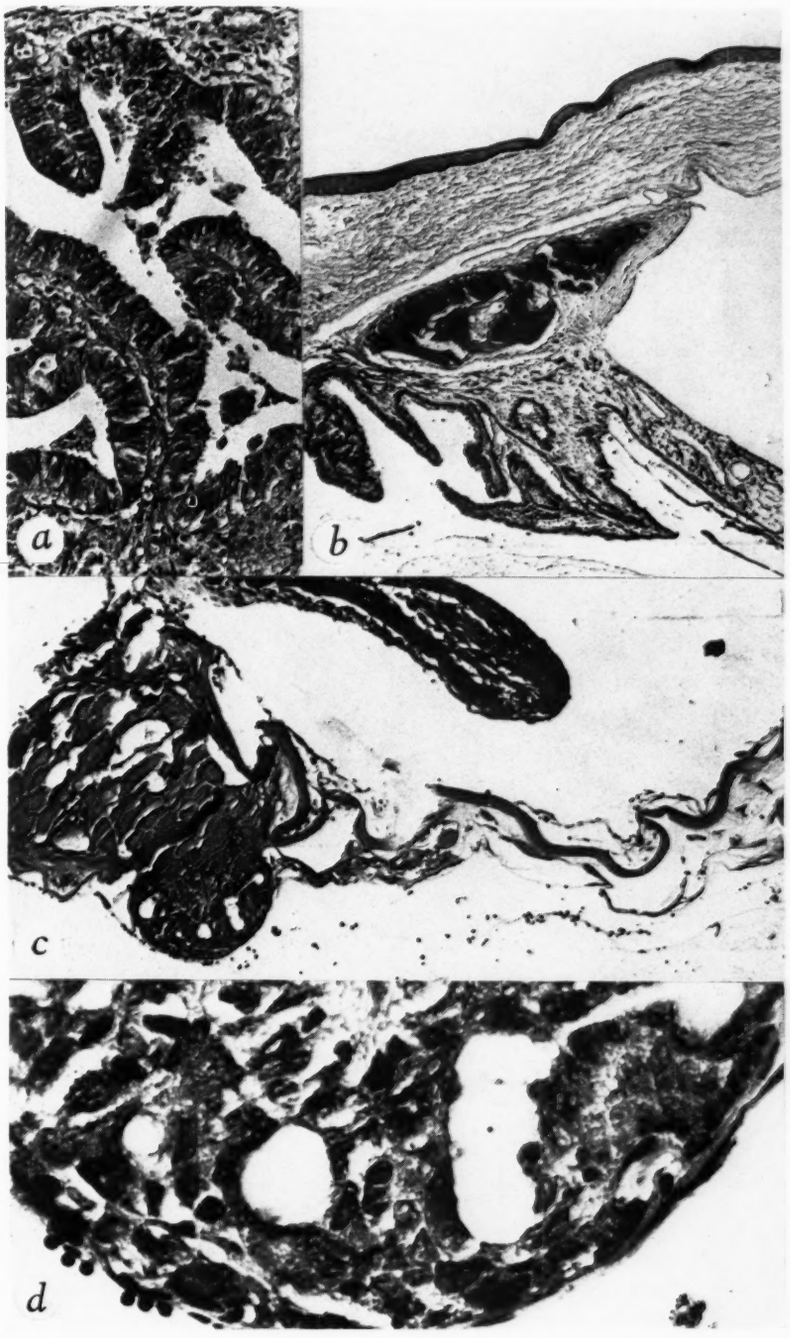


FIG. 2.—Tissue 2. *a*. A papillary adenocarcinoma of the endometrium (hematoxylin and eosin, ×120). *b*. Transplant of tissue 2, 68 days after transplantation into anterior chamber. Evidence of residual fibrosis, calcification and osteogenesis of tumor in the angle (hematoxylin and eosin, ×45). *c*. Intralenticular transplant of tissue 2, 68 days after transplantation. Most of the lenticular fibers have been extruded and absorbed, leaving a collapsed lenticular capsule. The tumor is just beneath the posterior capsule on the left (hematoxylin and eosin, ×80). *d*. Higher magnification of *c*. Note the very fine posterior capsule (hematoxylin and eosin, ×450).

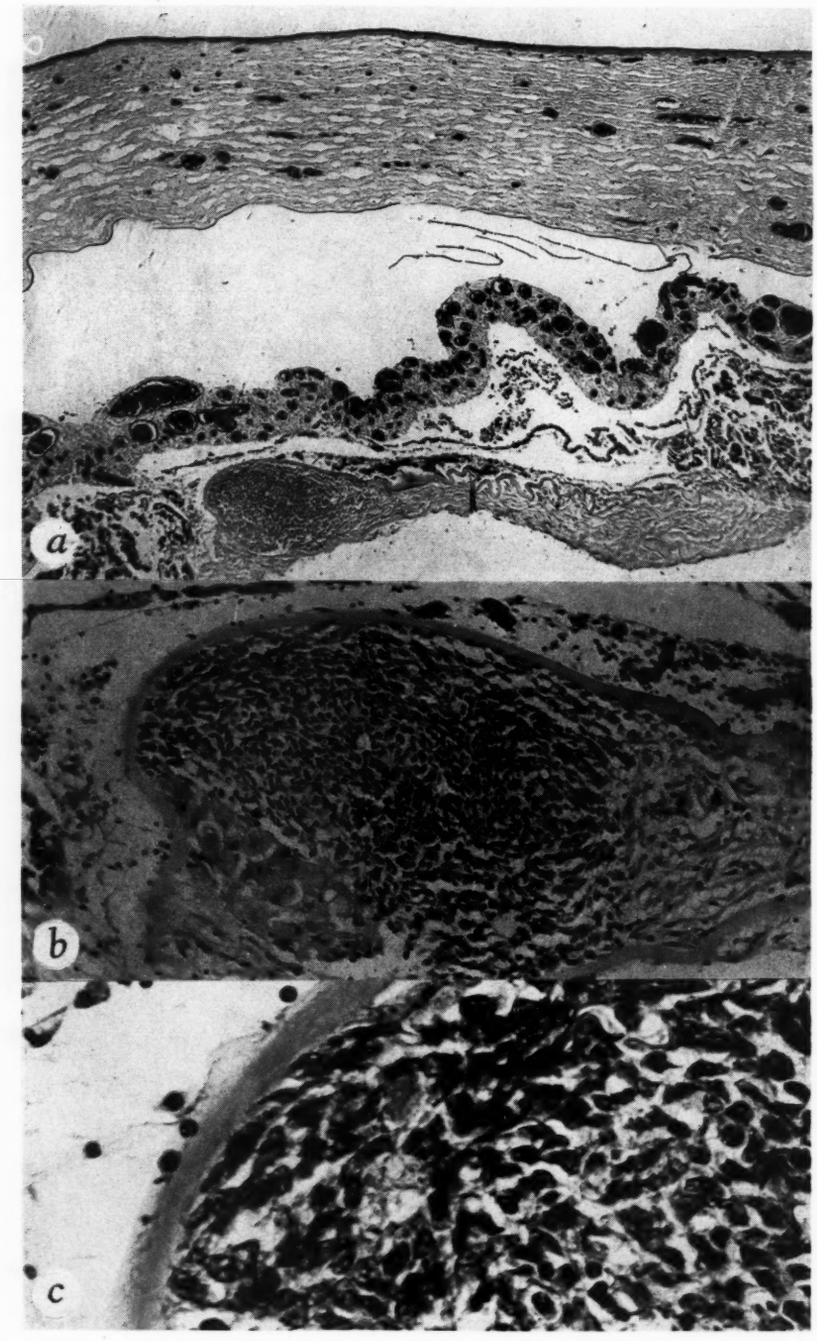


FIG. 3.—Tissue 3. A fibrosarcoma of the forearm. *a*. Intralenticular transplant thirteen days after transplantation. Most of the lenticular fibers have been extruded and absorbed. The clearly defined anterior capsule is protecting the tumor tissue from vascularization and cellular infiltration (hematoxylin and eosin, ×32). *b*. Higher magnification of *a*. Note avascularity of tumor transplant. There is some vacuolation of anterior capsular epithelial cells (hematoxylin and eosin, ×125). *c*. Higher magnification of *b* (hematoxylin and eosin, ×450).

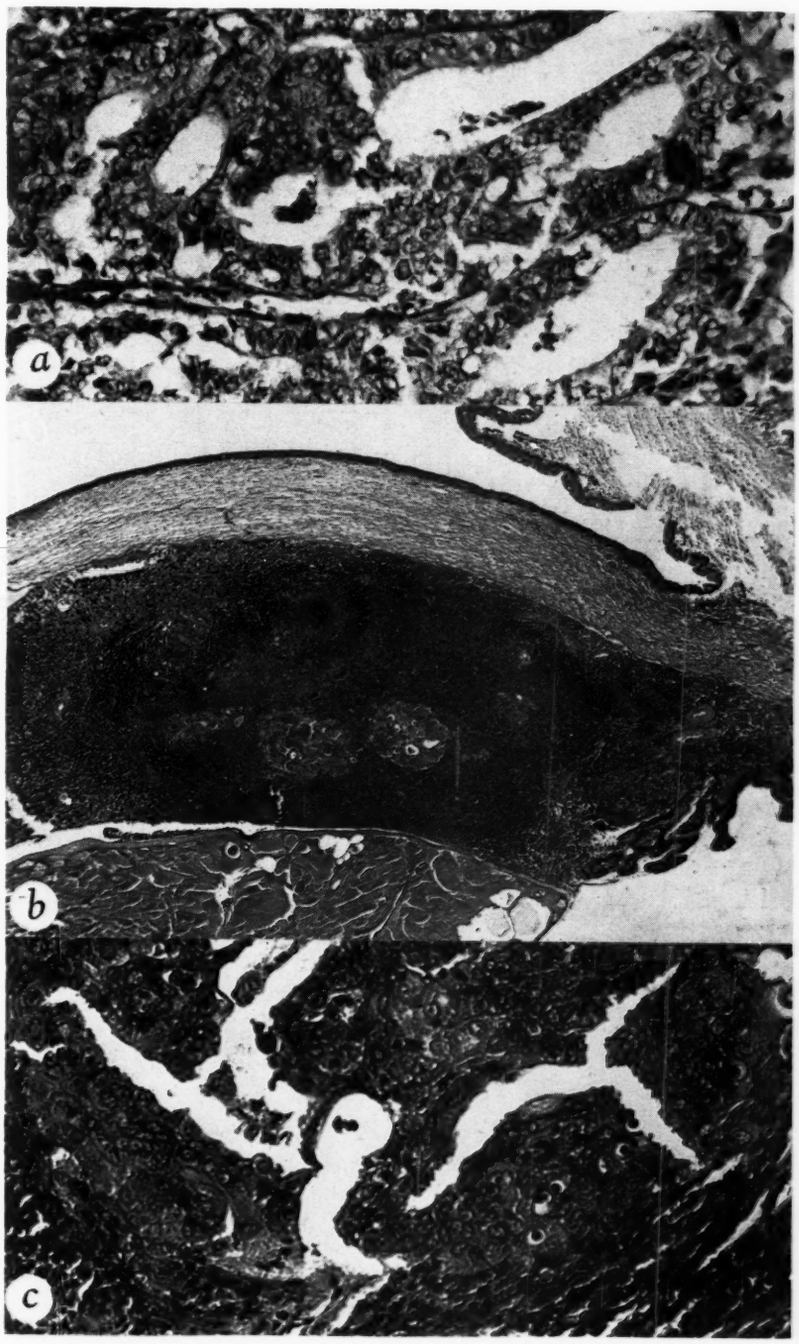


FIG. 4.—Tissue 4. *a*. An adeno-acanthoma of the ovary. More than 90 per cent of the tumor was composed of adenocarcinomatous tissue, as illustrated (hematoxylin and eosin, ×250). *b*. Transplant of tumor 4, 60 days after transplantation into anterior chamber. The anterior chamber is completely filled with an exudate of polymorphonuclear neutrophils and lymphocytes which surround several small islands of resistant squamous-cell carcinoma (hematoxylin and eosin, ×25). *c*. Higher magnification of *b* (hematoxylin and eosin, ×200).

FIG. 5.—Tissue 5. Normal bronchial elements: cartilage, mucous glands and ciliated columnar epithelium, as well as small-cell carcinoma. *a*. Section made from tissue from which portions for transplantation had been removed. *b*. Sixty-four days after intralenticular transplantation. An island of benign cartilage and ciliated columnar epithelium lie within the lenticular capsule. Note the characteristic sinuous conformation of a ruptured lenticular capsule and rolling of the free edges of the capsule. Practically all of the lenticular fibers have been lost. Scar tissue binds the damaged lens to the posterior surface of the cornea (hematoxylin and eosin, ×35). *c*. Higher magnification of portion of *b*. Note the benign ciliated epithelium (hematoxylin and eosin, ×800).

noma which was transplanted into the anterior chamber of an animal which died eighteen days later were destroyed. Lymphocytes, plasma cells, and a few foreign-body giant cells had infiltrated the remnant of the tumor (tumor 16). Three transplants of malignant tumors (tumors 6, 8, and 9) into the anterior chamber were completely absorbed within three weeks after transplantation.

## COMMENTS

The results of this study offer good evidence that heterotransplants of malignant tumors of human beings into the anterior chamber of the eye can seldom be accomplished. Opinions were based on the observation of 167 transplants of 40 malignant tumors. Eleven transplants of three benign tumors, four transplants of placental tissue, and four of normal bronchial tissue were also observed, but the tissue in each case was destroyed by the alien hosts.

The survival of benign as well as malignant heterologous tissues when transplanted within the lens was a unique observation. Although only five intralenticular transplants were observed, their survival suggests that similar results might be expected in a larger series. Successful heterologous intralenticular transplantation of normal tissue seems especially significant.

The fundamental difference between anterior chamber and intralenticular transplants was vascularization of the transplants into the anterior chamber as compared with the lack of vascularization of intralenticular transplants. Subsequent invasion of the transplants in the anterior chamber by lymphocytes, plasma cells, and occasionally foreign-body giant cells destroyed the transplant.

Lambert and Hanes (22) and Lumsden (26) demonstrated that tumors as well as normal tissue would grow in the plasma of alien species. These intralenticular transplants survived as *in vivo*, avascular tissue cultures nourished by imbibition of nutrient material from the intra-ocular fluids.

As indicated in the introduction and résumé of the literature, there have been many conflicting reports concerning heterologous tumor transplantation into the anterior chamber. Zahn (37), Herzog (16), Ruben (32), Woglom (36), and recently Lushbaugh and Steiner (27) were unable to achieve success, and the reports by Hegner and Keysser of successful heterotransplantation of sarcoma of human beings into the anterior chamber were brief and did not contain illustrations. They failed to achieve successful transplants of carcinomas. Furthermore, actively proliferating granulation tissue might be confused with sarcomas.

Since 1941 Greene (4–8) has made reports of successful heterotransplantation of several tumors from animals and human beings into the anterior chamber. Apparently metastasis has not occurred in the animals harboring heterotransplants of malignant tumors. On the other hand, metastasis has been described in animals harboring homotransplants of tumors in the anterior chamber (12, 14, 15, 20). It seems reasonable to expect metastasis to occur if vascularization of heterotransplants

TABLE 2

TISSUE FROM TUMORS OF HUMAN BEINGS TRANSPLANTED SOLELY INTO ANTERIOR CHAMBERS OF EYES OF GUINEA PIGS

| Tissue | Type of tissue | Number of transplants | Successful transplants |
|---|---|---|---|
| | Malignant tumors: | | |
| 6 | Squamous-cell carcinoma | 4 | 0 |
| 7 | Adenocarcinoma | 3 | 0 |
| 8 | Adenocarcinoma | 3 | 0 |
| 9 | Melanosarcoma | 5 | 0 |
| 10 | Myxosarcoma | 2 | 0 |
| 11 | Squamous-cell carcinoma | 5 | 0 |
| 12 | Fibromyxosarcoma | 3 | 0 |
| 13 | Adenocarcinoma | 3 | 0 |
| 14 | Adenocarcinoma | 4 | 0 |
| 15 | Adenocarcinoma | 4 | 0 |
| 16 | Seminoma | 4 | 0 |
| 17 | Transitional-cell epithelioma | 4 | 0 |
| 18 | Adenocarcinoma | 6 | 0 |
| 19 | Hypernephroma | 4 | 0 |
| 20 | Fibromyxosarcoma | 3 | 0 |
| 21 | Fibrosarcoma | 3 | 0 |
| 22 | Osteogenic sarcoma | 5 | 0 |
| 23 | Lympho-epithelioma | 6 | 0 |
| 24 | Adenocarcinoma | 4 | 0 |
| 25 | Adenocarcinoma | 5 | 0 |
| 26 | Transitional-cell epithelioma | 5 | 0 |
| 27 | Hypernephroma | 6 | 0 |
| 28 | Hodgkin's disease | 4 | 0 |
| 29 | Adenocarcinoma | 4 | 0 |
| 30 | Adenocarcinoma | 3 | 0 |
| 31 | Hypernephroma | 3 | 0 |
| 32 | Adenocarcinoma | 3 | 0 |
| 33 | Transitional-cell epithelioma | 3 | 0 |
| 34 | Adenocarcinoma | 4 | 0 |
| 35 | Fibrosarcoma | 4 | 0 |
| 36 | Adenoma of the bronchus | 6 | 0 |
| 37 | Adenocarcinoma | 6 | 0 |
| 38 | Squamous-cell carcinoma | 6 | 0 |
| 39 | Adenocarcinoma | 6 | 0 |
| 40 | Squamous-cell carcinoma | 6 | 0 |
| 41 | Transitional-cell epithelioma | 6 | 0 |
| | Benign tumors and normal tissue: | | |
| 42 | Parathyroid adenoma | 3 | 0 |
| 43 | Parathyroid adenoma | 4 | 0 |
| 44 | Cystadenoma lymphomatosa | 4 | 0 |
| 45 | Placenta | 4 | 0 |
| Total | | 170 | 0 |

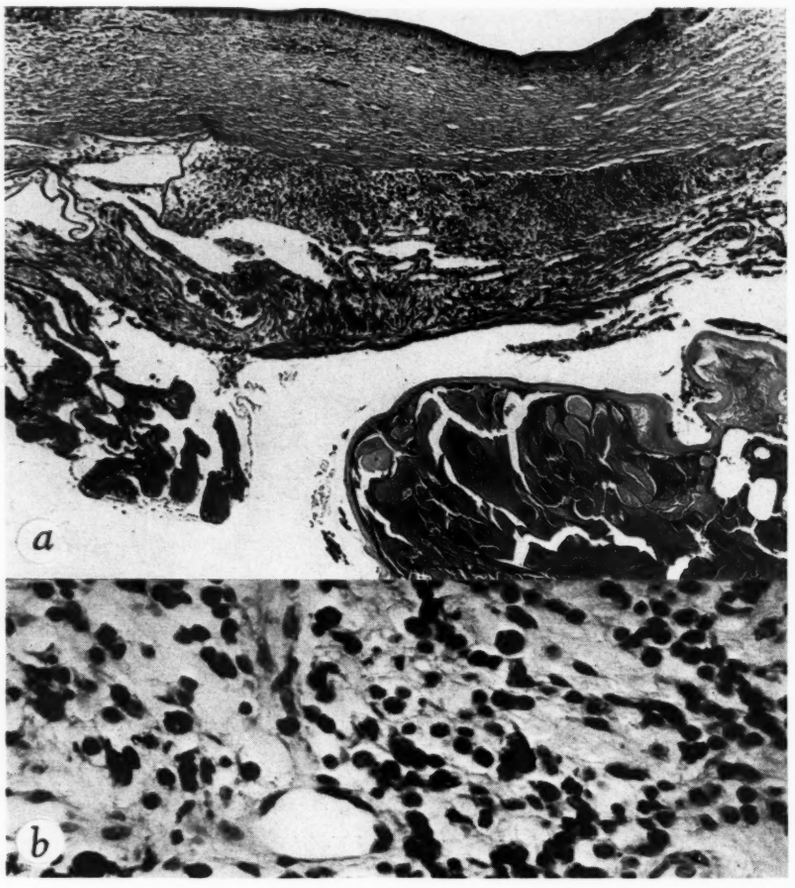


FIG. 6*a*.—Transplant of tissue 13, 60 days after transplantation into anterior chamber. The tumor cells have been completely destroyed. The remnant of the tumor is vascularized and infiltrated with plasma cells and lymphocytes (hematoxylin and eosin, ×50). *b*. Higher magnification of *a*. Note vascularization and character of cellular infiltrate in remnant (hematoxylin and eosin, ×400).

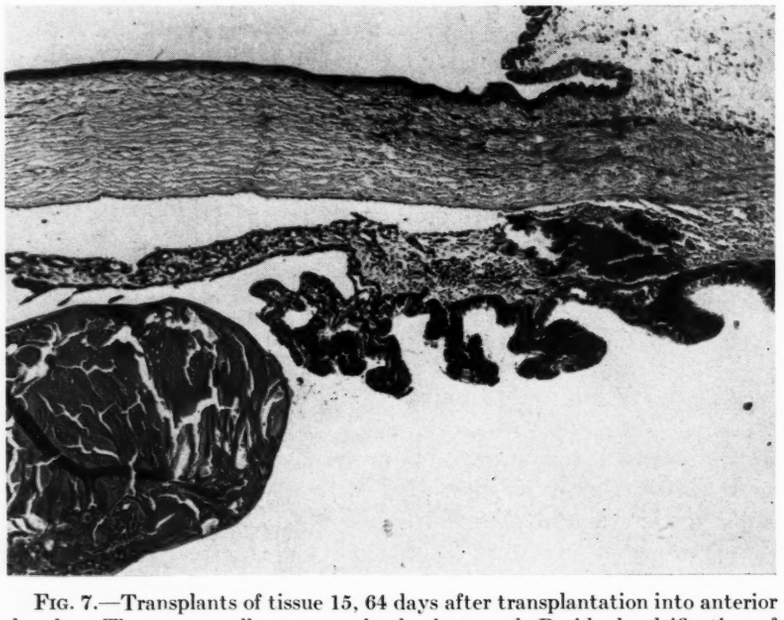

FIG. 7.—Transplants of tissue 15, 64 days after transplantation into anterior chamber. The tumor cells are completely destroyed. Residual calcification of some tumor remnant and lymphocytic infiltration of the ciliary body are present (hematoxylin and eosin, ×50).

of malignant tumors into the anterior chamber was successful.

It might not be amiss to suggest that investigators might re-examine their material in view of the results of intralenticular transplants reported herein. Masina (28) described a "pseudocapsule" around one of his transplants into the anterior chamber. This might represent the lenticular capsule. As indicated in the results, most lenticular fibers may be extruded, leaving only the capsule collapsed around some tumor tissue. Furthermore, this inclosed avascular transplant may be displaced to the vicinity of the angle.

Greene's criticism of the generally accepted methods of microscopic diagnosis of neoplasms hardly seems admissible. The evaluation of biologic characteristics of neoplasms based on histopathologic characteristics is a sound principle. Since the days of Virchow, pathologists have been correlating the behavior of tumors with their morphologic and cytologic characteristics and have thus established well-founded criteria for the evaluation of neoplasms. It is, of course, desirable for the pathologist to classify accurately all neoplasms studied. While it is occasionally difficult to classify accurately the highly malignant, completely undifferentiated tumors, still the biologic implications are clear in such instances to physicians and laymen alike. Even if infiltrative or metastatic tumors were transplantable to the anterior chamber of alien species, the clinical value of such a procedure would be limited. The present goal in diagnosis of tumors is to discover neoplasms while they are still largely or completely *in situ*, not after they are widely infiltrative or have given rise to distant metastasis.

The technic of intra-ocular transplantation is not difficult. The transplants into the anterior chambers were accurately placed with few exceptions. The tissue transplanted was carefully evaluated both by frozen sections before transplantation and by fixed sections of the tissue contiguous to the portions transplanted. The period of refrigeration was not excessive.

Evaluation of transplants by gross examination is unreliable.

The element of time has entered into several reports of heterotransplants. One animal died 1 week after transplantation of tissue into the anterior chamber. Transplantation should not be considered successful in this case even though early vascularization was evident. Although meager, the early results which were available suggested that between 1–3 weeks after transplantation, destruction of the anterior chamber transplant begins. There is a need for more early post-transplantation observations.

Greene has indicated that he has observed growth beginning several months after transplantation into the anterior chamber. In none of the transplants which were observed for several months was there any evidence of survival or growth of the transplant.

It seems reasonable to suggest that careful attention to the technic of transplantation and careful choice of material for transplantation should obviate the necessity of using unduly large numbers of animals in a series for a given tumor.

An instance of only partial survival incident to partial protection of a transplant by the lenticular capsule has already been cited. Another factor which might delay vascularization and reaction of the host to the heterotransplant is the possibility of the transplant's lying free and unattached in the aqueous humor of the anterior chamber for a period. Such an avascular transplant should also probably be considered an *in vivo* tissue culture and not a successful transplant.

These results indicate that successful heterotransplantation of tumorous tissue as a biologic test of malignant disease is not a feasible procedure.

## SUMMARY AND CONCLUSIONS

Transplants of 40 malignant tumors of human beings into the anterior chambers of 167 guinea pigs did not survive, with one exception. In this single exception partial survival of resistant squamous-epithelial elements occurred with a marked reaction of the host.

Transplants of four malignant tumors and one specimen of normal tissue from human beings within the lens of the eyes of five guinea pigs survived, and in at least one case the transplant grew. The lenticular capsule prevented vascularization, infiltration, and destruction of the intralenticular transplants which were nourished by imbibition of nutrient material from the intra-ocular fluids through the capsule. Thus the avascular, intralenticular transplants are tantamount to *in vivo* tissue cultures.

Intralenticular transplantation is probably of limited value in assessing the biologic characteristics of neoplasms, since these limited observations suggest that any heterologous tissue, benign or malignant, will survive within the lens.

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# Tobacco Smoking as an Etiologic Factor in Disease. I. Cancer*

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Clinicians seem to be in wide disagreement on the relationship between tobacco and cancer. Ochsner (1) and Lickint, (2), for example, considered smoking an important etiologic factor in cancer of the lung. Johnson (3) did not consider specifically the relation between smoking and cancer, but he believed that the only pathologic effect of heavy smoking is congestion of the pharynx. The clinician's point of view is well summarized by the statement of Cameron (4) that for every expert who blames tobacco for cancer of the lung, there is another expert who says tobacco is not the cause.

## METHODS OF STUDYING SIGNIFICANCE OF TOBACCO

*Clinical histories of individual patients.*—The diverse clinical impressions are based to a considerable extent on individual experience and on the clinical histories of individual patients. For example, Hermann (5) observed cancer of the vocal cords in a man 42 years old who smoked heavily and inhaled the smoke. In contrast, no tumor developed in an identical twin who did not smoke. Bogen and Loomis (6) report that bronchogenic carcinoma was found at autopsy in only one woman at the Olive View Sanitarium, and she had given a history of excessive smoking of cigarettes for more than 15 years. Friedell and Rosenthal (7) described eight patients who developed cancer of the mouth following the use of chewing tobacco.

*Smoking habits of patients with cancer.*—Individual histories are interesting and important in creating clinical impressions, but they only point the way toward studies on groups of patients. Many men have reported on the smoking habits of patients with a particular type of cancer.

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As early as 1915, Abbe (8) in New York reported on 100 private patients with cancer of the mouth. Of these, 90 were men, all of whom except one were heavy smokers. Nearly all of the men used three to twenty cigars a day. This surprisingly high percentage of cigar smokers may be attributed in part to two facts. First, the men were private patients who were presumably in good economic condition and who would be expected to prefer cigars rather than the cheaper cigarettes. Second, the study was made in 1915, when smoking habits of the general population were different from the present. In spite of these two factors, the number of heavy cigar smokers still seems to be unusually high.

Bloodgood (9) reported that nearly all patients with cancer of the tongue used tobacco to excess. He considered tobacco, in whatever form, an important etiologic factor. The literature on the smoking habits of patients with cancer of the lip and of the tongue was reviewed in detail by Haase (10). According to this author, the percentage of smokers among patients with these tumors varied from 100 to 10 per cent in different studies, with an average of 46 per cent. The wide variability in percentages throws doubt on the accuracy and the significance of the statistics.

Jackson and Jackson (11) reported in 1941 that 95 per cent of men with cancer of the larynx were smokers. In spite of this apparently high percentage of smokers, they (12) considered smoking only one of the minor causes of cancer of the larynx. They did not regard it as an essential cause.

Hermann (5) in Essen found that 26 out of 30 male patients (87 per cent) with laryngeal cancer smoked regularly, and most of them heavily; eighteen patients inhaled during smoking. Arkin and Wagner (13) in Chicago reported in 1936 that 90 per cent of 135 patients with cancer of the lung were chronic smokers. These workers, as many others, stress the etiologic significance of inhalation of tobacco smoke. Brockbank (14) in Man-

chester, on the other hand, found only 15 per cent heavy smokers and 21 per cent nonsmokers in 62 patients with pulmonic cancer. Grace (15) states that, in an unusually large series of patients with cancer of the lung, the individuals were heavy cigarette smokers, and almost all were inhalers.

Nearly all of these investigators in different cities report the high incidence of smoking among patients with cancer of the respiratory tract. The findings are suggestive, but the significance of these high percentages cannot be determined without knowing the smoking habits of a control group.

*Correlation of increase in smoking and increase in cancer.*—Some men used a third method of studying the etiologic significance of smoking. McNally (16) reported that in the United States the cigarette consumption was thirty-four times as much in 1930 as in 1905 (123.8 billion as compared to 3.7 billion). In contrast, the use of cigars declined by 17 per cent during this interval (from 7.6 to 6.3 billion). McNally attributed the reported increase in cancer of the lung to the observed increase in the use of cigarettes. Hoffman (17) also correlated the increase in pulmonic and esophageal cancer with the increased popularity of cigarette smoking. Many other writers, according to Hueper (18), considered that the greater incidence of cancer of the lung was due to the increase in smoking.

TABLE 1

FORM USED IN TAKING THE HISTORIES OF THE SMOKING HABITS OF PATIENTS

| | Smoking habits | | | |
|---|---|---|---|---|
| | Light | Moderate | Heavy | Duration |
| Cigarette | 10 or less | 10–20 | More than 20 | |
| Cigar | 2 or less | 2–4 | More than 4 | |
| Pipe | 3 or less | 3–6 | More than 6 | |
| None | | | | |

*Experimental studies.*—These clinical and statistical studies have led many investigators to approach the problem experimentally by studying the carcinogenic effect of smoke or of extracts of tobacco. It is not necessary in this report to review in detail the experimental work, especially since good summaries of the literature were given by Flory (19) in 1941 and Stern and Willheim (20) in 1943. It will suffice to mention the over-all findings of the work on animals.

Most workers obtained no effect or only slight proliferative changes in mice, rats, and rabbits subjected to tobacco tar or to tobacco smoke (21–23).

A few men obtained only a single carcinoma in their entire group of treated animals (24, 25).

In contrast, Roffo (26) painted the ears of rabbits with tobacco tar and derivatives from the tar and reported the development of many tumors which he diagnosed as carcinoma.

Flory (19) obtained tumors of the skin in 50 out of 53 rabbits and in 17 out of 242 mice treated with tar produced by destructive distillation of tobacco, and with tar obtained by smoking tobacco in pipes. In the mice, two tumors were diagnosed as squamous-cell carcinomas, and fifteen as papillomas. Most of the rabbits developed papillomas and a few "carcinomatoid" growths. The latter tumors invaded locally both connective tissue and lymphatic vessels, but they did not metastasize and did not have the property of unrestricted growth. These tumors were not, therefore, considered cancers.

The experimental evidence on the etiologic significance of tobacco in cancer is then equivocal. An editorial in *Lancet* (27) concludes with the understatement that the work with animals has not yet cleared cigarette smoking from suspicion as an etiologic factor.

*Smoking habits of patients with cancer and in control individuals.*—In the present work the smoking habits of patients with a particular type of cancer were compared with the smoking habits of men in a control group. Several investigators (28, 17, 29, 30) have used this method. Their work will be reviewed later and will be compared with the findings in this report.

## METHODS

In 1941, Dr. Baker started the practice at this hospital of having a clerk take a history of the smoking habits of each patient at time of admission by means of a questionnaire shown in Table 1.

In 1948, Drs. Ballard and Dolgoff summarized 5,003 records, selected at random, as to such factors as smoking habits and diagnosis. These men were particularly interested in the relation of smoking with peptic ulcer and heart disease. The data thus obtained were coded and placed on punch cards to facilitate analysis. The punch cards were prepared and handled by Mr. Vernon Graunke and Mr. G. O. Baldo, of Veterans Administration.

The 5,003 records are reviewed in this paper to determine the association of smoking and cancer.

It should be noted that the patients in this study were men, nearly all of whom were veterans of World War I or II or other wars. The patients were admitted during 1942–44.

## OBSERVATIONS

*All patients as a control group.*—In a study such as this, the crucial problem is the choice of a control group. Some factors to be considered in such a

problem have been discussed by Schrek and Allaben (31). One control group that might be used would be the entire group of 5,003 patients studied. By this means, the patients with any particular disease are compared with all the other patients. Let us first try this type of control group.

Cigarette smoking is the principal factor that is considered in this paper. It may be assumed that cancer would not be associated with, or be the result of, mild smoking. Therefore, cigarette smoking for this study was defined as the use of ten or more cigarettes a day. The duration of smoking was not considered at this point.

To compare the incidence of cigarette smoking in the different groups of patients, the graphic method described by Schrek (32) is used and is presented in Figure 1. Each point in the graph shows the number of patients and the percentage

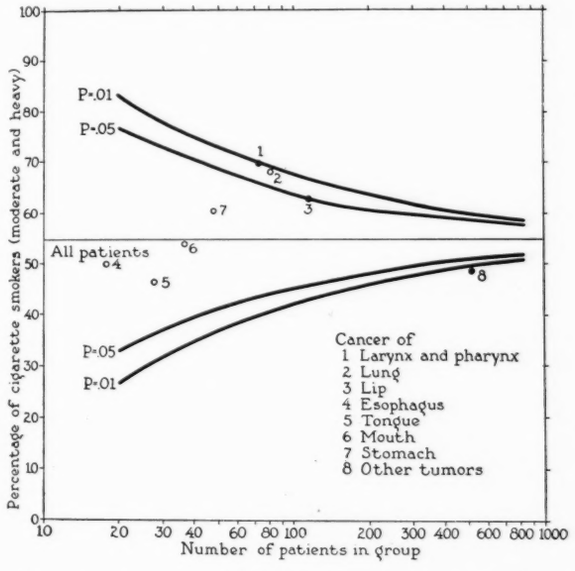


FIG. 1.—A comparison of the percentage of cigarette smoking in all patients with that in patients with cancer of certain sites. The percentage of smokers in the control group consisting of 5,003 patients is 54.8 per cent and is represented by the horizontal line. The curves show the limits of significance. A point on the graph that falls outside these curves differs significantly from the control in its percentage of cigarette smokers.

of cigarette smokers in the group. The percentage of smokers in the control group consisting of all 5,003 patients is 54.8 per cent and is represented by a horizontal line. The distance between the points and the horizontal line represents the difference between the percentages of smokers in each clinical group and the control. To determine whether this difference is statistically significant, curves have been drawn to show the limits of significance. A group of patients represented in the graph by a point that falls outside these curves may be said to differ significantly in its percentage of cigarette smokers from the control.

Of special interest in the graph is the group of patients with "other tumors," that is, tumors other than those of the respiratory and upper digestive tract. This large group of 522 patients had 48.8 per cent smokers, which was significantly lower than the 54.8 per cent for all patients.

The finding of an apparent negative correlation between miscellaneous cancer and cigarette smoking casts doubt on the validity of using all the patients as a control group. In fact, patients with cancer and with other diseases are not comparable in several respects. In the first place, there is the

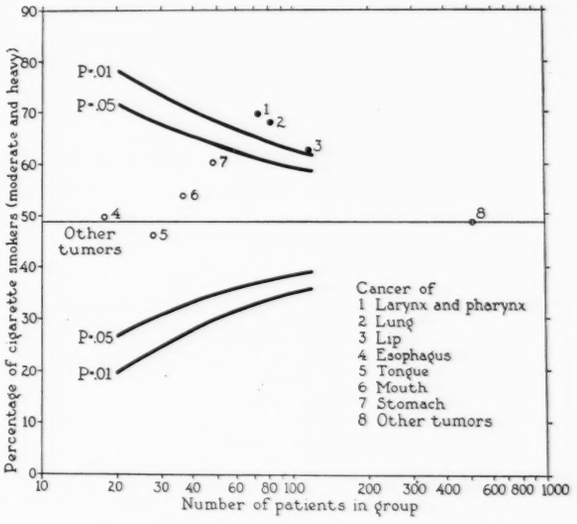


FIG. 2.—A comparison of the percentage of smoking in all patients with tumors with that in patients with cancer of certain sites. Explanation is the same as for Fig. 1.

probable difference in the age distribution of the patients in the two groups. Furthermore, one must take into consideration the fact that this hospital during 1942–44 not only was a general hospital but was, in addition, a cancer center. As a general hospital, patients from Chicago and the vicinity were admitted to it with all types of medical and surgical conditions. As a cancer center for the Veterans Administration, patients with tumors were referred to this hospital from other veterans' hospitals in the entire Midwest. Since smoking habits may be assumed to vary in different parts of the country, one cannot, at least in this hospital, use patients with other diseases as a control for patients with tumor.

It may be concluded, then, that, according to smoking habits, all patients were not a suitable control for comparing with tumor patients. A more suitable control group for studying patients with tumors of the respiratory and digestive tract would seem to be men with "other tumors."

*Patients with other tumors as a control group.*—In Figure 2, the control group was 522 men with other tumors, of whom 48.8 per cent smoked cigarettes to a moderate or greater extent. This percentage is represented by a horizontal line. As in Figure 1, curves are drawn to show the limits of significance. The percentages of smokers among patients with tumors of the stomach and mouth are higher than the control, but the differences are not statistically significant. This lack of correlation between the incidence of cigarette smoking and of tumors of the upper digestive tract may be due to an insufficient number of patients or to the absence of biologic relationship between smoking and the occurrence of these types of tumors.

In contrast, the groups of patients with tumors of the lung, larynx and pharynx, and the lip have a significantly higher percentage of smokers (68.3, 69.9, and 62.9 per cent, respectively) than the control groups of patients (48.8 per cent).

There is then a correlation between cigarette smoking and the incidence of cancer of the lip and of the respiratory tract. Is the correlation of biologic significance? Before considering this problem, one has to study in more detail the validity of the control group used.

*Racial distribution.*—In testing the validity of the control group, many factors can be considered. In this study only race and age were tested to determine whether these factors may be responsible for the correlation of cigarette smoking and cancer of the respiratory tract and lip.

The racial distributions of control and clinical groups are presented in Table 2, which shows the percentage of white patients in the different groups of men with cancer. For the control group, 93.1 per cent of the men were white. No significant variations from this percentage were observed for patients with tumors of the lung (89.0 per cent) and of the larynx and pharynx (94.5 per cent). Of the 116 patients with cancer of the lip, however, all or 100 per cent were white. This percentage is significantly higher than that for the control group.

Patients with cancer of the lip differed, then, from patients with "other tumors" in a higher percentage of white men. Is this racial difference in the clinical and control groups responsible for the relatively high percentage of cigarette smokers among patients with cancer of the lip? This question can be answered easily by excluding the colored patients from the control groups (Table 2). The white patients with cancer of the lip had 62.9 per cent cigarette smokers among them as compared to 50.0 per cent for the white men with other tumors. The difference in percentages may be considered as probably statistically significant ($P = 0.012$). In contrast, the groups of the white patients with cancer of the respiratory tract had significantly elevated percentages of cigarette smokers. It may be concluded that the differences in racial distribution are not responsible for the observed difference in the smoking habits of patients with cancer of the respiratory tract.

TABLE 2

NUMBER AND PERCENTAGE OF MODERATE AND HEAVY CIGARETTE SMOKERS IN WHITE AND COLORED MEN WITH CANCER

| LOCATION OF TUMOR | NO. OF PATIENTS | | | Percentage of white patients | Percentage of cigarette smokers in white patients | Percentage of cigarette smokers in colored patients |
|---|---|---|---|---|---|---|
| | Total | White | Negro | | | |
| Other sites | 522 | 486 | 36 | 93.1 | 50.0 | 33 |
| Lung | 82 | 73 | 8 | 89.0 | 71.2* | 50 |
| Larynx and pharynx | 73 | 69 | 4 | 94.5 | 69.6* | |
| Lip | 116 | 116 | 0 | 100.0* | 62.9† | |

* Percentage is significantly higher than the control percentage for "tumors of other sites" ($P < 0.01$).
† Percentage is probably significantly higher than the control percentage ($P = 0.01$–$0.05$).

Incidentally, it may be noted that the data of Table 2 can be tested to determine whether there is any difference in the percentage of cigarette smokers among white and colored patients. In men with "other tumors," for example, cigarette smoking was observed in 50 per cent of the white and only 33 per cent of the colored men. The statistical analysis shows, as might be expected, that colored men had a significantly low percentage of cigarette smokers.

*Age distribution.*—A second factor that has to be considered is age distribution in the clinical and control groups. A detailed analysis of the age distribution is presented in Figure 3. This graph shows the frequency distribution curves on arithmetic probability paper. This type of graph paper has the advantage of facilitating the comparison of several frequency distributions and permits the representation of a normal distribution by a straight line (Schrek, 33).

The age distribution of the 522 control patients with other tumors is represented in the figure by an irregular curve. The irregularity of the curve indicates that the age distribution is not normal and that the patients are a heterogeneous group. The heterogenicity arises from the fact that the patients in this hospital fall into three distinct categories, namely, veterans of World War I and II and of the Spanish-American War.

A second observation that can be derived from the figure is that the age distributions for the clinical groups differ to an appreciable extent from the control. These differences are greatest in the lower age groups and may possibly be attributed to varying numbers of World War II veterans in the groups.

The age distribution of the control and the clinical groups are also expressed in Figure 3 by means of the average age and the standard deviation. It was found that the arithmetic mean for the ages of the patients with cancer of the lung, larynx and pharynx, and lip (51.46, 52.57, and 52.72 years) do not differ significantly from that for the control group (51.98 years). In contrast, the standard deviation of the three clinical groups (5.98, 6.90, and 7.92 years) are definitely lower than that for the control (10.62). The age distributions for the three clinical groups differ then, at least in one respect, from that for the control.

Evidently, it is necessary to eliminate or minimize the variations in age distributions before comparing the smoking habits in the clinical and control groups. Table 3 presents a detailed analysis of the percentage of cigarette smokers in white patients. It may be concluded from this table that, even when age and race are controlled, the percentage of cigarette smokers in men with cancer of the larynx and pharynx is definitely higher than in the control group ($P = 0.01$). For cancer of the lung, the percentage of cigarette smokers is probably higher ($P = 0.03$).

An incidental observation is that in the patients with tumors in other sites, the older men 50–59 and 60–69 years have a lower percentage of cigarette smokers then men 40–49. This finding may be correlated with McNally's report (16) of an increase in cigarette consumption. The greater popularity of smoking might be expected to affect the younger men more than the older.

Other factors have to be considered in comparing the clinical and control groups. Inasmuch as the patients with tumors are derived from the entire Midwest and from city, town, and farm, it is necessary to consider the geographic distribution of patients. Unfortunately, data on this factor

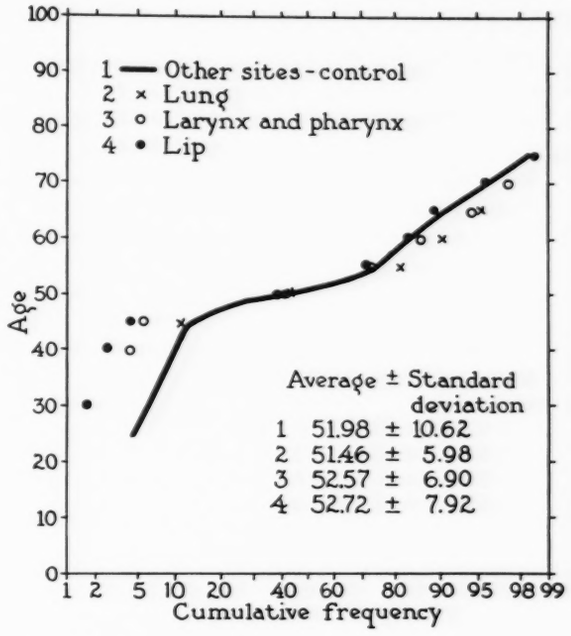


FIG. 3.—The age distribution of patients with cancer of various sites. The irregular curve represents the age distribution of the 522 control patients with other tumors. The irregularity of the curve indicates that the age distribution is not normal and that the patients are a heterogeneous group.

TABLE 3

PERCENTAGE OF MODERATE AND HEAVY CIGARETTE SMOKERS IN WHITE MEN ACCORDING TO AGE AND TYPE OF CANCER

| | No. of white men with tumors of | | | | Percentage of moderate and heavy cigarette smokers in white men with tumors of | | | |
|---|---|---|---|---|---|---|---|---|
| Age | Other sites | Lung | Larynx and pharynx | Lip | Other sites | Lung | Larynx and pharynx | Lip |
| 20– | 34 | 0 | 0 | 2 | 50 | | | 100 |
| 30– | 10 | 0 | 2 | 1 | 40 | | 100 | 0 |
| 40– | 171 | 35 | 24 | 47 | 57.9 | 77* | 58 | 70 |
| 50– | 192 | 30 | 33 | 48 | 49.5 | 67 | 82† | 56 |
| 60– | 62 | 8 | 8 | 14 | 40.3 | 63 | 38 | 71* |
| 70– | 17 | | 2 | 4 | 18 | | | 25 |
| *P* | | | | | | 0.03 | 0.01 | 0.06 |

* Percentage is probably significantly higher than the control percentage ($P = 0.01$–$0.05$).

† Percentage is significantly higher than the control percentage for "tumors of other sites" ($P < 0.01$).

were not obtained in reviewing the 5,003 original records. In previous studies in this hospital, Schrek and Allaben (31) found that the groups of patients with cancer of the lung and larynx had the same percentage of southerners and the same percentage of rural dwellers as in control groups. It is probable, then, that there are no significant differences in the geographic distribution of patients with cancer of the respiratory tract and of control patients. The previously observed correlation between the incidence of cigarette smoking and of cancer of the respiratory tract cannot be attributed to the extraneous factor of geographic distribution.

Schrek and Allaben (31) found that men with cancer of the lip had a significantly high percentage of individuals from rural sections (44 per cent as compared to 26 per cent for all cancer patients). The percentage of southerners among patients with cancer was slightly but not significantly higher (30 per cent as compared to 26 per cent). These differences in the geographic distribution of patients with cancer of the lip and of control patients are an additional reason for disclaiming any biologic significance for the high percentage of cigarette smoking in patients with cancer of the lip.

TABLE 4

THE DURATION AND THE AGE AT ONSET OF CIGARETTE SMOKING (MODERATE AND HEAVY) IN PATIENTS

| Location of tumor | No. of cases of heavy and moderate cigarette smoking | Age at onset of smoking | | Duration of smoking | |
|---|---|---|---|---|---|
| | | Average | Standard deviation | Average | Standard deviation |
| Lung | 56 | 21.3 | 6.7 | 29.2 | 8.3 |
| Larynx and pharynx | 51 | 21.8 | 8.6 | 30.7 | 7.9 |
| Lip | 73 | 19.7 | 6.5 | 31.2 | 9.4 |
| Other sites | 255 | 21.5 | 7.7 | 27.6 | 10.4 |

TABLE 5

CIGARETTE SMOKING IN PATIENTS WITH HISTOLOGICALLY PROVED TUMORS OF THE RESPIRATORY TRACT

| Site of tumors | No. of patients | Moderate and heavy cigarette smokers | |
|---|---|---|---|
| | | No. | Per cent |
| Lung: | | | |
| Histologically proved cases | 47 | 30 | 64 |
| Other cases | 35 | 26 | 74 |
| Total | 82 | 56 | 68 |
| Larynx and pharynx: | | | |
| Histologically proved cases | | | |
| Larynx | 43 | 31 | 72 |
| Pharynx | 5 | 4 | 80 |
| Larynx and pharynx | 3 | 2 | 67 |
| Nasopharynx | 13 | 8 | 61 |
| Total | 64 | 45 | 70 |
| Other cases | 9 | 6 | 67 |
| Total | 73 | 51 | 69 |

*Duration and age at onset of smoking.*—In some types of tumors it appears that the development of cancer in an individual depends not only on the carcinogenic agent but also on the age of the individual at the time of the first exposure to the agent. It is, therefore, of importance to investigate the age at which the patients started to smoke.

The history of the duration of smoking was obtained as indicated in the questionnaire in Table 1. Subtracting the duration of smoking from the age of the patient gave an estimate of the age at onset of smoking. It would have been preferable to have interrogated the patient directly as to his age at onset of smoking. There probably was a large percentage of error in the reported duration and in the derived age at onset.

Table 4 presents the average duration and the age of onset for patients in the clinical and control groups. Only the moderate and heavy cigarette smokers are considered in the table. It is seen that the average duration for the four groups of patients is approximately the same (27.6–31.2 years). The slight differences in the averages are not significant. The duration of smoking varied considerably (from 1 to 60 years). This wide range is reflected by the high standard deviations for the duration (7.9–10.4 years). There were no significant differences in the standard deviations. According to these results, the duration of cigarette smoking for patients with cancer of the respiratory tract is not appreciably greater than for patients with other tumors.

The average age at onset of smoking was approximately 21 years for the patients in both the clinical and the control groups. This average seems high, and it may indicate the unreliability of the method used in deriving the age at onset. The standard deviations for the four groups varied from 6.5 to 8.6 years and did not differ significantly from each other.

The present findings failed to confirm an anticipated longer duration of smoking and an earlier age at onset of cigarette smoking for patients with cancer of the respiratory tract.

*Histologic classification of tumors.*—Not all tumors in the clinical groups were examined by biopsy or autopsy. Table 5 shows the number of patients with histologically proved tumors and the smoking histories of these individuals. It is seen that of the 82 patients with cancer of the lung, more than half (47) had the diagnosis confirmed by biopsy or autopsy. The percentage of cigarette smokers in the proved and nonproved cases were

approximately the same (64 and 74 per cent, respectively).

Of the 73 patients with cancer of the larynx and pharynx, 64 had positive biopsies. The percentage of cigarette smokers in the patients with proved cancers was 70 per cent. It is to be noted that the number of patients with tumors of the pharynx and nasopharynx was so small that it is not possible to obtain satisfactory percentages of cigarette smokers.

*Complete smoking habits.*—Inasmuch as there appears to be a correlation between moderate and heavy cigarette smoking for cancer of the respiratory tract, it seems necessary to consider in detail the smoking habits of the patients with these tumors.

Figure 4 shows the percentage of mild, moderate (10–20 cigarettes a day), and heavy cigarette smokers for patients in the clinical and the control groups. It is seen that patients with cancer of the respiratory tract had a higher percentage of both heavy and moderate cigarette smokers than the control. In contrast, the group of men with cancer of the lip had a greater percentage of mild and moderate smokers. These findings lend support to the belief that the association between smoking and cancer of the respiratory tract has biologic significance, but the correlation between smoking and cancer of the lip is only incidental.

Figure 4 also compares the nonsmokers in the four groups. For cancer of the lung, of the larynx and pharynx, and of the lip, the percentages of nonsmokers were less than for other tumors (14.6, 13.7, and 10.3 per cent as compared to 23.9 per cent). This finding is not surprising. Since the previous finding was a positive correlation between cigarette smoking and cancer of the respiratory tract and lip, one would expect an associated negative correlation between nonsmoking and the three types of cancer.

Other factors that have to be considered are cigar and pipe smoking. Figure 5 indicates that patients with cancer of the respiratory tract had relatively low percentages of cigar and pipe smoking. These findings, as in the case of the previously observed low incidence of nonsmokers, are apparently the result of the high percentages of cigarette smokers in cancer of the respiratory tract.

## DISCUSSION

*Review of literature on use of control groups.*—The present findings are dependent upon the statistical method of setting up a control group to compare with the clinical group. This method was used for studying the relationship of smoking and cancer by several investigators.

Broders (28) observed that, in a group of 537 patients with cancer of the lip, only 11 were women. He compared the smoking habits of these patients with 500 control men. Both groups had approximately the same percentage of nonusers of tobacco (19.51 and 21.4 per cent). There was,

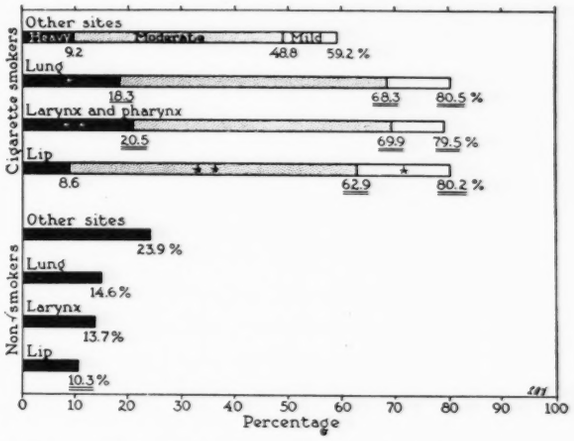


FIG. 4.—This shows the percentage of mild, moderate, and heavy cigarette smokers for patients with cancer of various sites.

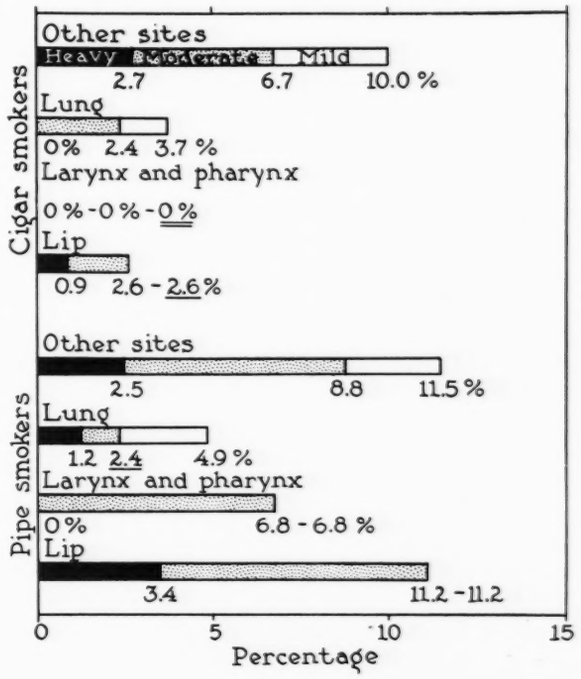


FIG. 5.—This shows the percentage of pipe and cigar smoking for patients with cancer of various sites.

however, a much higher percentage of pipe smokers among the smokers with cancer than in those of the control group (78.48 and 38.03 per cent) and a much lower percentage of cigarette smokers (1.16 as compared to 59.04 per cent in the control). The control group was not, however, entirely a satis-

factory one, as is indicated by the fact that the average age of the control men was 36.07 years, whereas the average age of the cancer patients was 57.3 years. It seems unfortunate that the remarkable differences in the smoking habits of the two groups cannot be properly evaluated because of an insufficient statistical analysis of the data.

In the present work, the group of patients with cancer of the lip did not have an elevated percentage of pipe smokers, nor a low percentage of cigarette smokers. The conflict in the findings do not necessarily mean that there is an error in either the work of Broders or in that of the present authors. Fashions in smoking vary at different times and among different groups of people. If smoking is an etiologic factor in cancer, the variations in the smoking habits will be reflected by changes in the type or the incidence of cancer. For the patients of Mayo Clinic in 1920, pipe smoking may have been a significant etiologic factor in cancer of the lip. For the patients of Hines Veterans Hospital in 1942–44, exposure to sunlight, rather than smoking, appeared to be the major etiologic factor in lip carcinoma.

Hoffman (17) studied the smoking habits of cancer patients in San Francisco, Buffalo, and Boston. He found that the percentage of heavy smokers was 67 among 27 patients with cancer of the lung, 43 among 37 with laryngeal cancer, and 48 among 120 with cancer of the lip. These percentages may be compared with 45.6 heavy smokers in 1,416 male patients with all tumors and 42.3 in 537 men with chronic disease. According to these figures, he failed to obtain any definite correlation between smoking and cancer of the respiratory tract. He minimized, however, this negative finding and attributed it to the small size of the groups, to the poor histories of the smoking habits, and to the presence of other etiologic factors. One also has to consider the suitability of the control group in considering the significance of the negative findings.

Striking results on the correlation of lung cancer and smoking were obtained by Müller (29), who compared the smoking habits of 86 men with cancer of the lung with 86 normal individuals in the same age groups as the patients. The group of patients had a relatively very high percentage of heavy smokers (50.0 as compared to 10.5) and a correspondingly low percentage of nonsmokers (3.5 as compared to 16.3). A heavy smoker was defined as one who used at least 7 cigars, 26 cigarettes, or 36 gm. of pipe tobacco. These findings led Müller to conclude that smoking is an important cause of cancer of the lung.

Recently, Wynder and Graham (30) reported to a cancer meeting in Memphis a comparison of the smoking habits of 200 male patients with cancer of the lung and of 500 control individuals. They found a relatively high percentage of heavy cigarette smokers (95.5 as compared to 50 per cent throughout) and a low percentage of nonsmokers (0.5 as compared to 11). A heavy cigarette smoker was defined as one who had smoked at least 20 cigarettes a day for 20 years. Wynder and Graham, like Müller, found a definite difference in the smoking habits of patients with pulmonic cancer and of control men.

A comparison of the results of the different investigators shows that Müller, Wynder and Graham, and the present authors agree in the finding that cancer of the lung is associated with heavy smoking.

An incidental observation that may be derived from the comparison is the difference in the smoking habits of the control groups of the various studies. For example, nonsmokers in the control groups were 21.4 (Broders), 3.5 (Müller), 11 (Wynder and Graham), and 23.9 per cent (present study). The observed differences are not surprising, since they only indicate that smoking habits vary in different localities and at different times. The finding stresses the need for determining the smoking habits of a suitable control group.

The work of Broders and the others indicates, furthermore, that it is not enough to use a control group for comparison with the cancer patients. It is also necessary to check carefully the control group to see that it is a suitable one for the particular factor under investigation.

*Is smoking an etiologic factor in cancer?*—From this study and from the work of Müller and Wynder and Graham, it may be concluded that there is a positive association or correlation of cancer of the lung and smoking, particularly cigarette smoking. The correlation is definitely statistically significant, but is it biologically significant? A statistical study cannot prove whether there is a cause-and-effect relationship between two factors. At best, the statistical study can provide circumstantial evidence that a correlation is biologically significant.

Willis (34) summarizes well the conclusions that may be drawn from a statistical study in his statement: "Comparison of the smoking habits of victims of lung cancer with those of control cases obtained by careful questionnaires, like [Müller's, afford strong grounds for suspecting the carcinogenic results of smoking; but, however strongly suggestive, they cannot afford incontrovertible proof—especially in the eyes of smokers themselves!"

The statistical findings are then only circum-

stantial evidence that smoking is an etiologic agent in cancer. Let us evaluate whether the evidence is valid.

The observed correlation between smoking and cancer can be explained in several ways. First, it may be considered that the correlation is a result of fortuitous or chance factors. The tests for statistical significance indicated to a large extent that the observed results are not due to the accidents of sampling.

A second explanation for the correlation is that it was not a direct relationship, but an indirect one and was dependent upon such secondary factors as race, age, occupation, etc. If it were possible to obtain a control group which was exactly the same as the clinical groups in all factors except two—the presence of cancer and smoking—it would be possible to test definitely whether there is a direct relationship between these two factors. Such a control group is frequently approached in experimental work, but rarely in clinical studies.

The chances of obtaining an indirect relationship dependent on secondary factors can be minimized to a considerable degree. The first step is to select the control group with a full and detailed knowledge of the local situation in regard to such factors as the type of patients admitted and the method of taking histories. In this study, for example, it was necessary to consider that cancer and noncancer patients were selected from different localities and populations and, therefore, noncancer patients could not be used as a control.

The second step in considering the possibility of indirect relationship is by testing and comparing the control and the clinical group in as many secondary factors as possible or as seem feasible. In this work it was possible to test age and race. An analysis of these two factors indicated that the control group was unsuitable for cancer of the lip but could be used for cancer of the respiratory tract. When age and race were equalized in the control and clinical groups, there still remained a statistically significant correlation between smoking and cancer of the lung and of the larynx and pharynx.

A third and important step in minimizing indirect relationships is the comparison of the statistical studies in different institutions by different investigators. The uniformity of the findings obtained by Müller and Wynder and Graham, and in the present study is impressive. There still remains a slight possibility that some abstruse secondary factor remained the same in all three investigations and resulted in an indirect relationship between smoking and cancer of the lung.

The correlation between smoking and cancer is, then, probably not due to fortuitous or secondary factors. It seems plausible, therefore, to formulate the hypothesis that there is a direct relationship between cigarette smoking and cancer of the respiratory tract and that cigarette smoking may be a carcinogenic agent.

There is another aspect to the problem. If cigarette smoking is a carcinogenic agent, how dangerous is this habit? This question could not be considered from the present data. Some information can be obtained, however, from other statistics and experiments.

In animal experimentation, application of a strong carcinogenic agent produces tumors in nearly all the animals, but a weak agent causes tumors in only a few animals.

It is well known that cigarette smoking is widespread. The control groups in the present study and in Wynder and Graham's work suggests that about half of the men are moderate and heavy cigarette smokers. According to the mortality statistics of the United States (35), 0.76 per cent of the deaths of men past 40 were due to cancer of the respiratory system. This relatively low percentage of deaths by cancer of the respiratory tract as compared to the high percentage of smokers indicates that smoking is, at most, only a weak carcinogenic agent.

## SUMMARY AND CONCLUSIONS

The smoking habits of 82 men with cancer of the lung and 73 men with cancer of the larynx and pharynx were compared with the smoking habits of a control group of 522 patients with miscellaneous tumors. A relatively high percentage of cigarette smokers was found among the patients with cancer of the respiratory tract, as compared to the control. This positive correlation between the incidence of cigarette smoking and the incidence of cancer of the respiratory tract appeared to be both statistically and biologically significant. There is strong circumstantial evidence that cigarette smoking was an etiologic factor in cancer of the respiratory tract.

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# The Use of X-rays To Determine the Mitotic and Intermitotic Time of Various Mouse Tissues*

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It has long been known that, as a general rule, the sensitivity of various normal and malignant tissues to x-ray is proportional to the amount of cell division which is occurring in the tissue. The number of mitoses seen in a biopsy of a tumor is one of the criteria for predicting the probable response of the tumor to irradiation (1). Since the amount of cell division, or more specifically the mitotic index, is dependent upon the mitotic and intermitotic times (5), a study was undertaken to determine which of these two factors is responsible for differences in the mitotic index seen in various tissues.

Many technics have been described by which the mitotic time of various cells can be determined. Hanging drop preparations (3, 8, 12) and tissue cultures (9, 13) have been used for direct observation of cell division. The mitotic and intermitotic times of some internal tissues have been established on the basis of the mitotic index and rate of growth of the tissue (2, 10). In the case of internal organs in which there is no increase in size, this method is not applicable. If, however, it were possible to determine the mitotic time of these tissues by some other means, then, knowing the mitotic index, the intermitotic time could be calculated (5) from the following formula:

$$\text{Intermitotic time} = \frac{\text{Mitotic time}}{\text{Mitotic index}}.$$

It has been shown that ionizing radiation causes a delay in cell division at early prophase (3, 4). At moderate dosage levels, those cells which are past this critical stage continue through mitosis with only slight, if any, slowing of the mitotic process (4). The slope of the downward trend of the mitotic index after irradiation must then be a measure of the mitotic time. In other words, one would expect cells to be completing the mitotic process, but no cells would be entering middle prophase. Theoretically, therefore, after a period equal to the mitotic time, there would be no mitoses present which were later than the ones blocked at early prophase. Actually, one would expect that a few cells would slip through the barrier at early prophase and also that a few cells which were past the barrier at the time of irradiation would be injured sufficiently to stop their progress through division (7)—the latter being manifested by various chromosomal aberrations such as clumped metaphases, chromosomal bridges of anaphase, etc. With the above theory in mind, a study was undertaken to determine the mitotic time of various mouse tissues by means of the fall in the mitotic index after irradiation.

## EXPERIMENTAL

$CF_1$ strain female mice, 8–16 weeks of age, which appeared healthy after 10 days' acclimation at this laboratory, were used in these experiments. Control animals were subjected to exactly the same handling procedures and confinement as the irradiated animals. The mice were irradiated in a flat lucite cage and allowed free movement during exposure. The radiation was delivered by a 250-kv. peak voltage x-ray machine with no filters. The inherent filtration of the x-ray tube was equivalent to about 2 mm. of aluminum. The dosage delivered to each group of mice was determined by means of a 300-r Victoreen ionization chamber placed at the center of the cage.

To study the effect of x-rays on the bone marrow, eight groups of seven mice were carried through the following procedure. Five animals were irradiated with 400 r of x-ray given at the rate of 100 r per minute. Animals were killed by crushing the cervical spine at 6, 12, 18, 24, and 30

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minutes after exposure. The control animals were killed before and after the irradiated mice. A specimen of bone marrow was removed from the femur by inserting a needle into the medullary cavity and forcing out the marrow. The specimen was mixed with a drop of fresh blood serum (human or rat) on a clean glass slide and a bone marrow smear prepared. This was then air-dried, stained with Wright's stain, and mounted in Canada balsam. The preparations were read to determine the number of mitoses per 5,000 nucleated cells. Each mitosis was classified according to its stage of division and its series, whether erythrocytic or myelocytic. Sample counts were performed on each bone marrow preparation to determine the relative number of white blood cells and nucleated red blood cells, so that a normal mitotic index could be calculated for these two kinds of cells.

TABLE 1

BONE MARROW

CHANGE IN MITOTIC ACTIVITY AFTER 400 r OF X-RAY

MITOSES PER 5,000 NUCLEATED CELLS

| Time after exposure | Myelocytes | Nucleated red blood cells |
|---|---|---|
| control | 11.2±1.1 | 8.4±1.1 |
| 6 min. | 8.0±1.7 | 5.4±1.0 |
| 12 min. | 8.4±1.1 | 4.1±0.7 |
| 18 min. | 6.8±1.2 | 3.6±0.8 |
| 24 min. | 3.9±0.9 | 3.2±0.6 |
| 30 min. | 2.7±0.9 | 2.0±0.8 |

$$\text{Standard error} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n (n - 1)}}$$

To determine the mitotic time of various other mouse tissues, 72 mice were divided into three groups of 24 animals each. These groups were given 0, 50, and 200 r of x-ray, respectively, at a rate of 50 r per minute. Eight animals from each group were sacrificed at 10, 20, and 30 minutes after the midpoint of exposure, or, in the case of control animals, the midpoint of confinement. The tissues to be studied were removed in a rapid manner (11) and in a definite order (6). The ears were removed first and placed in 0.5 per cent acetic acid; then the right inguinal lymph node, a segment of jejunum, the right adrenal gland, and the right ovary were removed and placed in Bouin's fixative. The epidermis was separated from the inner surface of the ears, after 24 hours in acetic acid at a temperature of 8° C., and 6-$\mu$ sections were prepared from the other tissues after routine histological precedures.

The mitotic activity of each tissue was then determined by the methods previously described (6). The number of mitoses per 100 standard fields, as outlined by a Whipple disc, and the number of cells per field were determined for the epidermis and the lymph nodes, thereby making it possible to calculate the normal mitotic index. The number of mitoses per 100 crypts of Lieberkühn and the average number of cells per crypt were determined for the jejunum. In the adrenal gland, the number of mitoses found in the zonae glomerulosae and fasciculatae was enumerated for 15 sections. Representative sections were then projected on white paper, and the outer border of the zona glomerulosa and the inner border of the zona fasciculata were outlined. The area of these two portions of the gland was determined by the use of a planimeter. After counting the number of cells in the zonae glomerulosae and fasciculatae of four of the adrenal glands which had been projected, it was possible to determine the number of cells per unit area of the projection. The number of mitoses per unit area and the normal mitotic index were then calculated. The mitotic index of the ovarian follicles was determined by counting the number of mitoses per 50 follicles with diameters of 50 $\mu$ or more and then finding the average number of cells per follicle.

In an effort to standardize the technic of counting mitotic figures, a cell was considered to be in mitosis only between the time, in prophase, of elongation of the chromosomes before the breakdown of the nuclear membrane; and the time, in telophase, of complete separation of the daughter cells. This definition of mitosis was very satisfactory for all tissues except the epidermis, in which the nuclear structure was distorted slightly by treatment with acid; therefore, the time of breakdown of the nuclear membrane was considered the beginning of mitosis.

## RESULTS

There was a fairly uniform decrease in the mitotic activity of the myelocytes and nucleated red blood cells after irradiation (Table 1).

A straight line, calculated to fit the experimental points for the mitotic activity in the myelocytes (Fig. 1), intersects the time ordinate at about 35 minutes. In the case of the nucleated red blood cells (Fig. 2) there is a flattening of the curve at the 24- and 30-minute points. If these points are neglected, and a straight line is calculated to fit the 0-, 6-, 12-, and 18-minute points, this line intersects the time ordinate at about 29 minutes.

The results obtained in the other five tissues are shown in Table 2. The responses seen in the jejunum, ovary, and lymph node are almost identical (Figs. 3, 4, and 5). If the slope between the 20- and 30-minute points for the 200-r dosage is used to determine the mitotic times of these three tissues, they are found to be 24, 21, and 23 minutes, respectively. The differences between the mitotic

times are well within the standard errors which were determined by mathematically deriving the formula for the line between the 20- and 30-minute points and by determining the intersections with the normal mitotic activity and with the time ordinate. The results in the epidermis and adrenal gland are more difficult to interpret. If the 20- and 30-minute points on the 50-r dosage curve are used to calculate the mitotic time of the epidermis, the resultant time is 30 minutes with a relatively large standard error (Table 3). The data for the epidermis from the 200-r dosage are definitely conflicting. The large standard error at the 20-minute point in the 200-r data makes it difficult in the case of the adrenal gland to be accurate in establishing the mitotic time. Judging from the shape of the curves obtained from the other tissues, the mitotic time is probably 20 or more minutes, rather than the figure of 14.4 ± 4.5 minutes. A tabulation of the mitotic times of the seven mouse tissues which were studied is presented in Table 3.

The mitotic index, as determined experimentally for each tissue, is shown in Table 3. It is evident that the crypts of Lieberkühn of the jejunum, the myelocytes, the nucleated red blood cells, and the follicles of the ovary have the higher mitotic indices—the crypts of Lieberkühn of the jejunum, for example, have about one cell in mitosis out of every 100 resting cells, as compared to two out of 10,000 in the zonae glomerulosae and fasciculatae of the adrenal gland.

The intermitotic times were calculated from the experimentally derived mitotic times and the mitotic indices (Table 3). Since the mitotic times of all seven tissues are relatively constant, the intermitotic time is inversely proportional to the mitotic index. Those tissues with the lower mitotic indices have the longer mitotic times. Of the tissues studied, the jejunum has the shortest intermitotic time (43 ± 8 hours) and the adrenal the longest (probably more than 1000 hours).

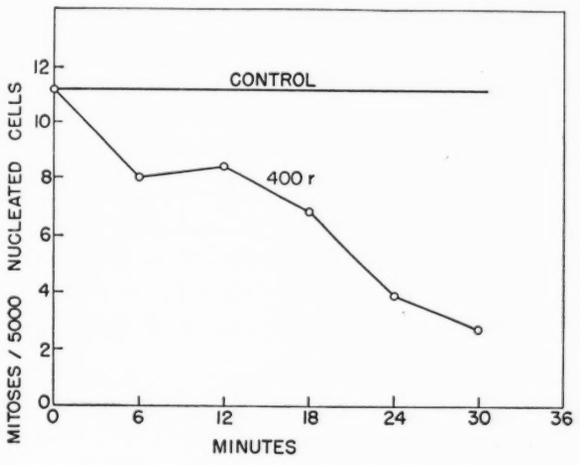


FIG. 1.—Bone marrow; myelocytic series

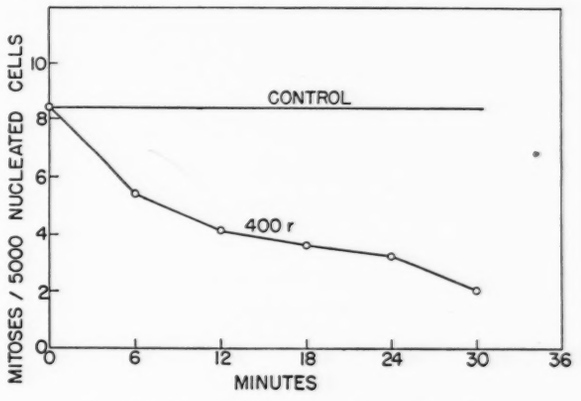


FIG. 2.—Bone marrow; erythrocytic series

TABLE 2

CHANGE IN MITOTIC ACTIVITY AFTER IRRADIATION

| TISSUE | DOSAGE IN ROENTGENS | CONTROL | TIME AFTER EXPOSURE 10 min. | 20 min. | 30 min. | CELLS PER CRYPT, FOLLICLE OR UNIT AREA |
|---|---|---|---|---|---|---|
| Jejunum (Mitoses/100 crypts) | 50 | 79.4±3.1 | 75.3±4.1 | 69.0±6.8 | 56.1± 9.3 | 85.7±1.8 cells/crypt |
| | 200 | | 74.6±8.5 | 53.5±8.2 | 20.3± 2.9 | |
| Ovary (Mitoses/50 follicles) | 50 | 55.6±5.2 | 57.2±6.0 | 49.9±5.9 | 45.5±11.4 | 390±44 cells/follicle |
| | 200 | | 50.8±7.1 | 37.9±6.7 | 11.5± 3.6 | |
| Lymph node (Mitoses/unit area) | 50 | 23.5±1.4 | 28.2±3.4 | 20.0±1.7 | 16.3± 1.8 | $(351\pm37)\times10^2$ cells/unit area |
| | 200 | | 20.8±1.4 | 16.4±1.3 | 6.3± 1.3 | |
| Epidermis (Mitoses/unit area) | 50 | 15.4±2.8 | 14.6±2.6 | 12.3±1.8 | 7.2± 1.7 | $(205\pm2)\times10^2$ cells/unit area |
| | 200 | | 17.3±2.8 | 4.7±1.2 | 8.9± 1.7 | |
| Adrenal gland (Mitoses/unit area) | 50 | 17.7±2.1 | 12.7±4.0 | 7.6±0.9 | 9.6± 3.2 | $(8.0\pm0.7)\times10^4$ cells/unit area |
| | 200 | | 19.7±2.1 | 20.4±4.2 | 8.1± 1.0 | |

## DISCUSSION

It would appear from the experimental data that the mitotic time of various types of cells can be determined by delaying mitosis in early pro-

phase by means of x-ray. In all seven types of cells which were studied there was a definite downward trend found within the first 30 minutes after irradiation. The slopes obtained for five of the

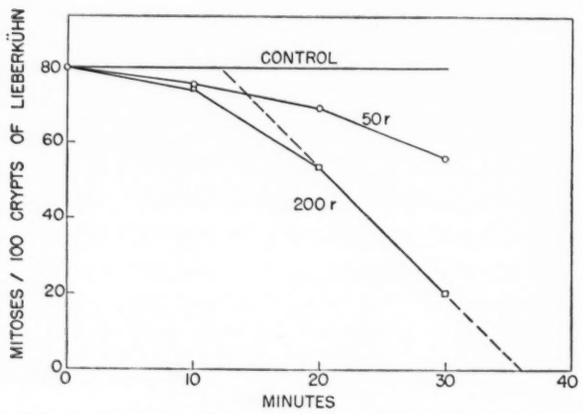


FIG. 3.—Jejunum: Mitoses in the crypts of Lieberkühn

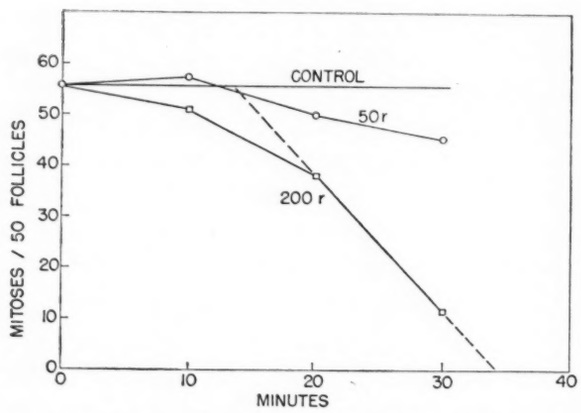


FIG. 4.—Mitoses in the Graafian follicles of the ovary

seven tissues (the crypts of Lieberkühn, the follicles of the ovaries, the lymph nodes, the nucleated red blood cells of the bone marrow, and the epidermis) were so comparable that the derived mitotic times varied only from 21 to 30 minutes. The myelocytes apparently have a slightly longer mitotic time; and the cells of the zonae glomerulosae and fasciculatae in the adrenal gland might possibly have a slightly shorter one, although the data for this tissue are difficult to interpret. Thus, it would appear that the mitotic times for the seven different tissues are quite comparable, probably varying between 20 and 35 minutes.

The observation that the mitotic activity in the jejunum, ovary, and lymph node did not drop significantly during the first 10 minutes after exposure can perhaps be accounted for by the criteria used to define mitosis. The beginning of mitosis was arbitrarily defined as the time when the chromosomes were elongated in prophase. If radiation blocked mitosis at a point about 10 minutes prior to this, there would be a number of cells still entering the arbitrarily defined beginning of mitosis equal to the number forming daughter cells, and the mitotic index would remain constant for this length of time. After ten minutes there would be no more cells entering the stage of chromosomal elongation in prophase; but there would still be cells completing division, and therefore the mitotic index would begin to fall.

It is apparent from the discussion above that

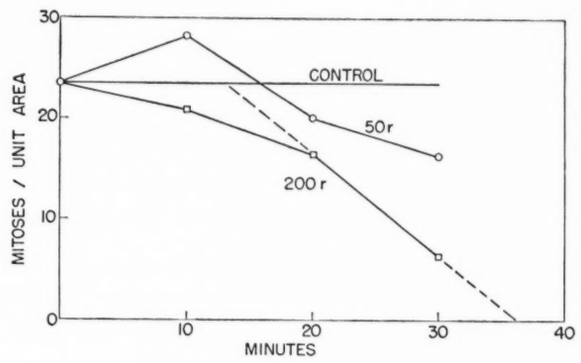


FIG. 5.—Mitoses in the lymph nodes

TABLE 3

MITOTIC INDEX, MITOTIC TIME, AND INTERMITOTIC TIME OF MOUSE TISSUES

| TISSUE | MITOTIC TIME IN MINUTES | MITOTIC INDEX | INTERMITOTIC TIME IN HOURS |
|---|---|---|---|
| Jejunum | 23.9± 4.5 | (9.3 ±0.4 )$10^{-3}$ | 43± 8 |
| Nucleated red blood cells | 29.5± 2.4 | (5.0 ±0.7 )$10^{-3}$ | 99± 16 |
| Myelocytic series | 35.3± 3.0 | (3.8 ±0.4 )$10^{-3}$ | 155± 20 |
| Ovary | 21.1± 4.8 | (2.8 ±0.4 )$10^{-3}$ | 123± 33 |
| Lymph node | 23.2± 3.3 | (6.7 ±0.8 )$10^{-4}$ | 580±110 |
| Epidermis | 30.2±12.0 | (7.5 ±1.4 )$10^{-4}$ | 670±300 |
| Adrenal gland | 14.4± 4.5 | (2.21±0.33)$10^{-4}$ | 1090±430 |

the mitotic time which was measured was only the period of time from the elongation of the chromosomes in prophase to the separation of the cytoplasm of the daughter cells. In the jejunum, ovary, and lymph node the delay in the fall of the mitotic activity might be a measure of the time in prophase that is sensitive to radiation and the elongation of the chromosomes. For these three tissues this time would appear to be 10–15 minutes.

Since the mitotic index is measured on the basis

of the criteria for mitosis mentioned above, and the mitotic time is also derived from the same arbitrary definition of mitosis, these two artificially introduced errors cancel out in the determination of the intermitotic time. Thus, the value of the average time between divisions is a true one and does not have arbitrary limitations.

## CONCLUSIONS

A method of determining the mitotic time of various tissues of the mouse by means of estimating the effect of x-ray on mitosis has been presented. The mitotic time was found to vary about 20–36 minutes in the seven tissues studied. The normal mitotic index and the mitotic time of these tissues were used to calculate the intermitotic time from the formula:

$$\text{Intermitotic time} = \frac{\text{Mitotic time}}{\text{Mitotic index}}.$$

It was concluded that, since the mitotic times were relatively constant as contrasted to the intermitotic times, the differences in the mitotic indices of the various tissues were due primarily to the variations in the intermitotic times. Thus, the mitotic index is inversely proportional to the intermitotic time, and, in general, tissue-sensitivity to x-ray increases, the shorter the intermitotic time.

Further experiments are being planned to study the mitotic and intermitotic times of certain mouse and rat tumors with the purpose of detecting any differences between normal and malignant types of cells.

## SUMMARY

A method has been proposed to determine the mitotic time of tissues, utilizing the effect of x-ray on cell division. The mitotic times of seven types of cells of the mouse were determined by this method. The intermitotic time was calculated from the experimentally derived mitotic time and mitotic index. Differences in the mitotic indices of tissues were found to be due primarily to variations in the intermitotic time.

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# Announcements

REVIEW ARTICLES: Although the primary purpose of *Cancer Research* is the prompt publication of original observations on the fundamental aspects of the cancer problem, review articles have appeared from time to time. The interest in reviews has increased with the acceleration of research in this field. Accordingly, *Cancer Research* will accept selected critical reviews on subjects dealing with cancer and allied fields. Such reviews should attempt to correlate the various approaches to a problem in a manner that will indicate the trends and emphasize the aspects that require further clarification. Critical analyses of the pertinent issues and expressions of opinions are encouraged. Preferably the reviews should not exceed five pages (4,500 words) in length, including references.

COMMENTS TO THE EDITOR: A limited space will be available for the publication of comments on published data in the cancer field. Comments written for the presentation of new data will not be published. It may not be possible to publish all comments sent to *Cancer Research*, and the Editorial Board will have the final decision on this matter. Comments should not exceed 500 words.

RADIOISOTOPE TECHNICS COURSES: Some vacancies still remain for the winter series of three Radioisotope Technics courses offered by the Special Training Division of the Oak Ridge Institute of Nuclear Studies.

Interested persons who can attend a course during the winter months are urged to make application for one of these courses in view of the heavy load of applications from university people for participation in the summer courses. The courses will begin on January 2, January 30, and March 6, 1950. Applications should be made as soon as possible. Requests for application and additional information should be addressed to Dr. Ralph T. Overman, Chairman, Special Training Division, Oak Ridge Institute of Nuclear Studies, P.O. Box 117, Oak Ridge, Tennessee.

The courses are offered at Oak Ridge to acquaint research workers with the safe and efficient use of radioisotopes as tracers. Emphasis is placed on individual laboratory work, with sufficient lecture material included to provide the necessary background information. Eleven of the courses already have been given. Thirty-two participants can be accommodated in each of the three four-week courses.

## ANNOUNCEMENT REGARDING AVAILABILITY OF DAMON RUNYON CLINICAL RESEARCH FELLOWSHIPS

The American Cancer Society announces the availability of Damon Runyon Clinical Research Fellowships. These fellowships, inaugurated last year, again are made possible by a grant from the Damon Runyon Memorial Fund for Cancer Research to the American Cancer Society. They are administered by the Society upon recommendation of the Committee on Growth of the National Research Council. In most cases a fellowship will provide a period of training in a hospital under the guidance of a qualified clinical investigator. However, fellowships may be awarded for training in a basic science provided that such training is directed toward preparing the fellow for clinical cancer research. They are limited to men and women holding the M.D. degree. It is emphasized that they are intended to promote training in clinical cancer research rather than in cancer diagnosis and therapy.

Applications submitted prior to March 1, 1950, will be acted upon during April. Fellowships approved at that time may become effective July 1, 1950, or at such other time as will meet the convenience of the fellow and the institution. Communications should be addressed to the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.

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**STATEMENT OF THE OWNERSHIP, MANAGEMENT, CIRCULATION, ETC., REQUIRED BY THE ACT OF CONGRESS OF AUGUST 24, 1912, AS AMENDED BY THE ACTS OF MARCH 3, 1933, AND JULY 2, 1946**

Of CANCER RESEARCH, published monthly at Chicago, Illinois, for October 1, 1949.

State of Illinois, County of Cook, *ss.*

Before me, a Notary Public in and for the State and county aforesaid, personally appeared S. W. Findley, who, having been duly sworn according to law, deposes and says that he is the Office Manager of The University of Chicago Press, publisher of CANCER RESEARCH, and that the following is, to the best of his knowledge and belief, a true statement of the ownership, management (and if a daily, weekly, semiweekly, or triweekly newspaper, the circulation), etc., of the aforesaid publication for the date shown in the above caption, required by the act of August 24, 1912, as amended by the acts of March 3, 1933, and July 2, 1946 (Title 39, U.S. Code, Section 233), to wit:

1. That the names and addresses of the publisher, editor, managing editor, and business managers are:

Publisher, The University of Chicago Press, 5750 Ellis Ave., Chicago 37, Ill.

Editor, Dr. Harold P. Rusch, McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.

Managing Editor—None.

Business Manager—None.

2. That the owner is: (If owned by a corporation, its name and address must be stated and also immediately thereunder the names and addresses of stockholders owning or holding 1 percent or more of total amount of stock. If not owned by a corporation, the names and addresses of the individual owners must be given. If owned by a partnership, or other unincorporated firm, its name and address, as well as those of each individual member, must be given.)

The American Association for Cancer Research, Inc., Massachusetts General Hospital, Boston 14, Mass.

Dr. Joseph C. Aub, Massachusetts General Hospital, Boston 14, Mass.

3. That the known bondholders, mortgagees, and other security holders owning or holding 1 percent or more of total amount of bonds, mortgages, or other securities are: (If there are none, so state.)

There are none.

4. Paragraphs 2 and 3 include, in cases where the stockholder or security holder appears upon the books of the company as trustee or in any other fiduciary relation, the name of the person or corporation for whom such trustee is acting; also the statements in the two paragraphs show the affiant's full knowledge and belief as to the circumstances and conditions under which stockholders and security holders who do not appear upon the books of the company as trustees, hold stock and securities in a capacity other than that of a bona fide owner.

5. The average number of copies of each issue of this publication sold or distributed, through the mails or otherwise, to paid subscribers during the 12 months preceding the date shown above was: (This information is required from daily, weekly, semiweekly, and triweekly newspapers only.)

The University of Chicago

S. W. FINDLEY, Office Manager of The University of Chicago Press, Publisher

Sworn to and subscribed before me this 10th day of October, 1949.

DARIS G. PEYER

Notary Public in the State of Illinois.

(My commission expires July 5, 1953) Residing in Cook County.